TEXTBOOK OF
Cytology
SECOND EDITION

WALTER V. BROWN

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TEXTBOOK OF
Cytology SECOND EDITION

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with 470 illustrations

THE C. V. MOSBY COMPANY
SAINT LOUIS 1974

PREFACE

Since the publication of the first edition of this book in 1969, cytology in the broad sense, which includes much of cell biology, has continued to advance, slowly in some areas but rapidly in others. In virology, knowledge of chemical and structural variability of the pseudoorganisms, assembly, relations to membranes, and host relations has progressed rapidly. New techniques for staining the heterochromatins of chromosomes and their uses in cytogenetics, especially human cytogenetics, is a new and fast-moving field of cytology. Knowledge of peroxysomes as a class of cytoplasmic organelles, under various names, has expanded considerably. The Kranz syndrome of certain green plants, which includes a new and specialized (although variable) photosynthetic pathway, is also a new and dynamic field of interrelated biochemistry, cytology, and anatomy. The study of microfilaments has taken on new significance in protoplasmic movements of all kinds. The discovery of "reverse transcriptase" has redirected some thinking in the field of replication-transcription-translation models. These are merely samples of recent cytological progress included in this second edition.

As discussed more fully in the preface to the first edition, emphasis continues on the broad and general treatment and the structural aspects of cytology, although the amount of biochemistry has generally been increased. The topics of history and nature of cytology, cell chemistry, and differentiation have been eliminated as specific discussions, and the glossary has not been included. Furthermore, the topics of cytogenetics and reproductive cells have been reduced and combined into one chapter. On the other hand, a new chapter on viruses and Prokaryota has been added, and such topics as ribosomes, mitochondria, microtubules, peroxysomes, and microfilaments have been considerably expanded. All chapters have been brought up-to-date, which required sometimes much and sometimes little modification of the first edition, depending on the amount of recent progress in the specific field.

Again we acknowledge our debt to all the scientists who have built cytology into the biologically fundamental and dynamic field that it is and to all the others, including those who have constructively criticized the first edition. We are especially indebted to our wives, who have aided one way or another in bringing this second edition to completion.

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CHAPTER 1

Cytological techniques

Cells and tissues must be prepared properly for the study of their morphological and chemical organizations. Most often, for a particular type of study to be undertaken, special techniques and procedures are needed to produce the proper results. In fact, knowledge of the chemistry of fixation is most important in the preparation of tissue for cytological studies. This is particularly true for histochemistry, both histochemistry per se and enzyme histochemistry. One must be certain in the case of enzyme histochemical studies that the fixatives used do not appreciably destroy or remove the enzyme activity or mask the reactive sites. Similarly, the proper preparation is equally important for the demonstration as well as identification of various chemical components within the cell.

Generally two avenues of approach can be used for cytological studies: living cells or tissues can be used, or the cells can be killed by chemical fixation.

The study of living cells can be best accomplished by the use of phase-contrast microscopy. At times the light microscope can be used, particularly if vital stains such as trypan blue, Janus green B, neutral red, etc., are used. These stains not only make the cells discernible, but many of the vital stains presently available will also selectively stain specific structures.

Killing the cells by chemical fixation preserves the morphology. After fixation, various specific staining procedures can be used.

FIXATION

Although fresh cells or tissues can be studied by phase-contrast microscopy, living tissues deteriorate rapidly, and permanency cannot be maintained for future reference. In addition, many cytoplasmic and nuclear structures may be ill defined in the living cell, but they can be studied more precisely when stained to produce better definition and contrast. As a result, the tissues used for cytological and histological studies are prepared to overcome some of the disadvantages of living tissues. Preparation of tissue also allows for some degree of permanency.

The most important single step in the preparation of tissues is fixation. Without proper precaution at this stage, alterations in cytological structure can render the tissue unsuitable for subsequent study. Fixation should accomplish the following things:

1. It should kill the cells rapidly so that at the time of microscopic examination it should be reasonably similar to the tissue at the time of killing.
2. It should alter the refractive indices so that the cell organelles and cellular inclusions can be readily seen.
3. It should harden the tissue somewhat so that after embedding the tissue can be cut readily.

At the present time there are numerous kinds of fixatives that can be used. The choice will depend on the nature of the study. For convenience, fixation can be divided into chemical and physical fixation.

Chemical fixation

Chemical fixation is the most commonly used method for the preparation of tissue for cytological as well as histological study. One of the prerequisites of chemical fixatives is that they must rapidly penetrate the tissue to produce the fixation of proteins and/or lipids. Many chemicals, for example, mercury and chromium salts, are excellent fixatives; however, their low rate of penetration in a pure form makes them unsuitable. On the other hand, acetic acid is a rapid penetrant but a poor fixative. Commonly, acetic acid is used in conjunction with a heavy metal salt. The advantage is that the acetic acid can act as a vehicle for the transportation of the heavy metal compound so that excellent fixation can occur. There are a large number of fixatives available for general cytological work as well as for more specialized work in cytochemistry.

Formalin. Formaldehyde, alone or in mixtures, is probably the most widely used chemical for fixing tissue. It does not appreciably shrink or swell the tissues. Its reactions in the tissue are numerous and complex. It can combine with a large number of different kinds of functional groups and, in so doing, can form methylene bonds. For the most part the reaction results from its addition to a compound containing an active hydrogen atom to form hydroxymethyl compounds (French and Edsall, 1945) as follows:

$$RH + HCHO \rightleftharpoons R{-}CH_2OH$$

This can further react with another compound to produce a methylene bridging between the two:

$$R{-}CH_2OH + R'H \rightleftharpoons R{-}CH_2{-}R' + H_2O$$

The $-CH_2-$ represents the methylene bridge. According to French and Edsall a number of compounds are available for reaction with aldehyde; this is called *formation.* They include amino, imino, amido, peptide, guanidino, hydroxyl, carboxyl, and sulfhydryl groups, and a number of aromatic compounds. It can readily be seen that with such diversity of reactions numerous cross-linkages can occur between protein groups and readily bind them together to produce good fixation. In addition, it is important to remember that washing after fixation can disrupt many of these bonds and open up reactive groups for histochemical studies.

Usually a 10% solution is used for fixation. This would be roughly a 4% absolute formaldehyde solution, since commercial formaldehyde is only 38% to 40% concentrated. Wolman (1955) studied the reaction of formation and maintained that a neutral or slightly alkaline solution produces the best fixation. At that pH there is rapid depolymerization of formalin to produce the monomers, and the monomer exists in the more hydrated form known as methylene glycol, $CH_2(OH)_2$, which is much more efficient as a fixative.

Several other aldehydes have been utilized for fixation. Of particular importance is glutaraldehyde. Not only is the substance good for general fixation but it is also an excellent fixative for electron microscopy. It has an additional quality of not interfering with enzyme sites for subsequent study in enzyme histochemistry (Sabitini et al., 1963). Glutaraldehyde is a five-carbon compound containing two aldehyde groups. It is perhaps for this reason that there is increase in cross-linking between protein groups to produce excellent fixation.

Metal ions. Metal ions include those heavy metals that act as protein precipitants. In general, only three metal salts are widely used in cytology—mercury, chromium, and osmium ions.

Mercury. Mercury salts such as the bichloride are sometimes used for fixation. In this type of fixation the mercuric ion

(Hg^{++}) is important because this bivalent ion can combine with two different proteins and bridge them (Wolman, 1955). In this reaction they can combine with carboxyl and hydroxyl groups as well as with amines. One interesting aspect is the high affinity for the sulfhydryl group. If a small quantity of mercury salt is used, it will bind the sulfhydryls in preference to other groups (Pearse, 1960).

Chromium. Chromium salts combine with water to form Cr—O—Cr complexes, and these complexes can combine with the reactive groups in protein (Pearse, 1960). According to Wolman, chromium fixation involves two stages—the primary, which can be reversed by washing, and the secondary, in which the bound chromate group is reduced to a chromic group. In general, the initial reaction involves carboxyl groups and to a lesser extent the amino group. The primary reaction between the chromium complexes and carboxylic group is followed by coordination with amino and hydroxyl groups (Green, 1953).

Osmium. As a tissue fixative, osmium (O_8O_4) is not recommended because of its low rate of penetration. However, its importance lies in fixation for electron microscopy, and therefore its mechanism of action has been studied. It is known that osmium tetroxide is a strong oxidizing agent and is reduced by unsaturated fats to produce a black precipitate. In fact, osmium can be used to detect fat in histochemical studies because the reduced osmium will appear black. Its mechanism of action (Porter and Kallman, 1953) consists of oxidation of the fatty acid double bonds by osmium to produce a monoester. Furthermore, osmium will also react with aliphatic hydroxyl (1,2-glycols), sulfhydryl, and amino groups (Wolman, 1955).

In general, the nuclei are not well preserved after osmium fixation. According to Porter and Kallman, osmium is excellent for cytoplasmic fixation. The proteins are quickly gelated, and it may form polymers with protein, establishing a linkage at double bond positions.

Unfortunately, overfixation may produce soluble end products that can be washed from the cell.

Alcohols and acetone. Alcohols and acetone are valuable in enzyme histochemistry because in general they leave the enzyme group available for subsequent reactions. The great disadvantage of alcohol fixation is the morphological disturbance produced. Little is known of the mode of action of alcohols, except that they denature proteins and dissolve many organic molecules.

Acetic acid. Acetic acid precipitates nucleoprotein and dissolves most of the cytoplasmic proteins. In general, acetic acid fixes those proteins in which the isoelectric point is near the pH of the acid. The bound water about the protein disappears. There is loss of electric charge, the protein molecules can form new cross-linkages, and the reactive groups of the proteins move close together (Wolman, 1955).

In retrospect. Of the large number of fixatives available, only the most common have been mentioned. However, when special studies are initiated, other fixatives or combinations of fixatives are used. These will be mentioned later. From an examination of the theory behind the various fixatives mentioned, it is evident that chemical fixation involves linking by the fixative to other molecules in the cell so that with subsequent treatment the molecules are held in position. Furthermore, in the process of fixation the globular proteins may be involved. Generally this entails (1) the unfolding of the globular protein molecule, which results in (2) the increased availability of heretofore masked reactive groups that cannot react with the fixative or remain untouched and become available in subsequent histochemical methods.

It can also be noted that with certain fixatives the binding capacity of acid and basic dyes may be affected. In general, those fixatives (formalin, for example) that

affect the basic groups shift the isoelectric point to the acid pH, which results in decrease of eosinophilia. The reverse also holds true with fixatives that bind the acid group (chromate fixatives); they render the tissue less basophilic.

Physical fixation

The physical methods of fixation include freeze-drying, frozen section, and freeze substitution. In all these methods the temperature of the tissues is lowered to a point where all or at least nearly all of the metabolic activity has ceased. Although these methods have been classed as fixing processes, it is doubtful whether they are such in the true sense. When the tissue is brought back to normal temperature, the metabolic activity commences again.

Freeze-drying. Freeze-drying is one of the best methods for the preservation of tissue. Not only is the morphological detail maintained but the enzyme systems are preserved. The freeze-dry procedure consists of several steps. The first step is quenching the tissue by very low temperature. This is brought about rapidly by immersing the tissue in a Dewar flask containing liquid nitrogen or in isopentane cooled in liquid nitrogen. The latter method has received wide acceptance because the isopentane acts as a good heat transfer, whereas the liquid nitrogen has an insulating effect and may delay the freezing of the tissue. (The insulating effect is due to the formation of nitrogen gas immediately around the warm tissue, and it is the gas that is supposed by many to have the insulating effect.) After the quench the tissue is placed in a tube or container to which a high-vacuum system has been applied. The temperature of the container is maintained at about -40° C during the drying period. The high degree of vacuum and the cold temperature allow the sublimation (evaporation from the solid state) of water molecules from the tissue. At the end of the process the tissue is preserved but is devoid of all water except that which is bound. After the drying period the tissues are generally embedded in paraffin or in Carbowax. The chief restriction of this technique is the length of time required to process the tissue (48 to 72 hours).

Frozen section. If tissues are frozen rapidly, the frozen water will serve as an embedding medium, and then sections can be cut if maintained at a suitable low temperature. This procedure is widely used for enzyme histochemistry and for routine studies in histology and cytology. Its advantage is the rapidity with which sections can be processed and the lack of chemical fixation which makes the method excellent for subsequent enzyme studies. Despite the advantages obtained, there are the following disadvantages to be coped with or at least to be aware of.

1. Freezing followed by thawing may possibly produce loss of architectural integrity. This is minimized by rapid cooling to prevent large ice crystals from forming and by placing sections into a fixative or incubation medium at the instant of thawing.
2. There can be loss of either the soluble enzyme or cofactors.
3. Diffusion of enzymes in unfixed tissues can lead to false positive results (Gomori, 1952). To prevent loss by diffusion the frozen sections can be placed in a fixative with a low freezing point such as acetone or ethanol to ensure some denaturation of the protein, which will, in many cases, fix the enzyme in postion and yet not inactivate them appreciably.

Generally the tissues are frozen with Dry Ice or cold carbon dioxide gas. The knife of the microtome used for cutting sections is likewise cooled to about the same temperature as the tissues. A refrigerated unit to cut frozen sections (cryostat) is now available. This unit has the advantage of maintaining the environment about the tissue and knife at the same low temperature, al-

lowing more efficiency in cutting sections (Fig. 1-1).

Freeze substitution. Excellent morphological results are obtained with freeze substitution. In addition, many of the enzymes remain active for enzyme histochemical studies. Basically, freeze substitution consists of two steps: quenching of the tissue and extraction of the water at a low temperature. Isopentane cooled in liquid nitrogen, liquid nitrogen, ethanol at –65° C, an acetone-Dry Ice mixture at –60° C, or propylene glycol can be used as the quenching agent. Water can be extracted at low temperatures ranging from –20° to –75° C, depending on the technique or reagents used. There are also many substitute reagents. A few that we have used are ethanol, ethanol-methanol (usually in a 1:1 ratio), and osmium-acetone. Other investigators have used propylene glycol, Rossman's fluid, and ethanol with a metal salt (Pearse, 1960).

After dehydration the tissues are allowed to reach room temperature and are then embedded.

METHODS IN CYTOLOGY

Fluorescence microscopy

Fluorescence microscopy has become an important research tool in cytology and histology. A high degree of accuracy is obtained, the techniques are relatively simple, the cost is low, and the time necessary to complete the studies is relatively short when compared to autoradiography. In essence, the use of fluorescence microscopy involves the conjugation or labeling of a specific substance such as protein, serum antibodies, etc. with a suitable fluorochrome. At the present time a number of fluorochromes are available. The choice depends on the nature of the investigation. These include fluorescein isocyanate, fluorescein isothiocyanate, DANS (1-dimethylamino-naphthalene-5-sulfonic acid), lissamine, and rhodamine RB200. The tagged proteins or antibodies are examined under ultraviolet or ultraviolet-blue light. The sites of localization are indicated by visible fluorescent transmission, the color of which depends on the fluorochrome used.

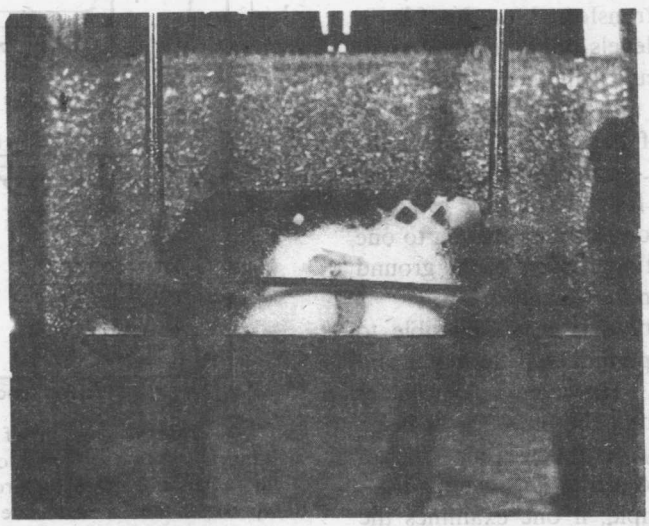


Fig. 1-1. Interior of a cryostat showing tissue (center) and microtome knife (below). The white material surrounding the tissue is the mounting medium. In the cryostat the environment is kept constant, thereby keeping both the tissue and the blade at the same low temperature.

Nature of fluorescence. To understand better the use of fluorochromes in cell research it is important to understand the theory by which fluorescence is accomplished. When a fluorochrome molecule absorbs a quantum of sufficient energy, it becomes excited, resulting in changes in its electron distribution. In many compounds this increased amount of energy is quickly dissipated to the surrounding molecules. However, in the case of fluorochrome the excited molecule is sufficiently stable so that it may return to ground state level and in so doing emit radiation. It is this usually visible emission that is called fluorescence, and its wavelength is always longer than the exciting radiation (Stokes's law).

This is visualized graphically in Fig. 1-2, in which *G* represents the ground state level and *E* represents the excited state level that results when the molecules absorb an exact amount of photon energy. The horizontal line at each level represents the vibrational state of the molecules. In general, when at equilibrium in their surroundings, these molecules are at the lowest of these levels *(E* and *G).* The transition *(A)* represents the absorption of a specific quantum by the molecule that is transferred to one of the upper vibrational levels of the excited state. The excited molecule will then usually lose vibrational energy and reach the lowest vibrational energy of the excited state. If the state is stable for approximately 10^{-8} seconds, it is possible for the molecule to lose a quantum of radiation in the transition to one of the higher vibrational levels of the ground state. This results in fluorescence.

At the present time it is not possible to predict which compounds will fluoresce and which will not. However, Pringsheim (1949) suggested that the presence of fused aromatic rings may be at least one characteristic that is involved in producing fluorescence. For example, if one examines the structure of rosamine, which has a fluorescent characteristic, and compares it with malachite green, a nonfluorescent compound, a striking similarity is seen, the difference being, however, the presence of an oxygen bridge in rosamine and its absence in malachite green (Fig. 1-3). The oxygen bridging no doubt imparts rigidity to the molecule, which may be at least one requisite for fluorescence.

Autofluorescence. Many structures within the cell as well as some intercellular structures undergo fluorescence autonomously when merely examined under ultraviolet light. Most common is a blue to blue-green autofluorescence. Some cytoplasmic granules will at times produce a moderate to strong yellow fluorescence. Most of the cytoplasmic fluorescence is concentrated in the mitochondria. In addition to autofluorescence due to molecular structure, other inclusions also produce an autofluorescence; for example, ceroids produce a yellow to yellow-brown fluorescence, whereas calcium pro-

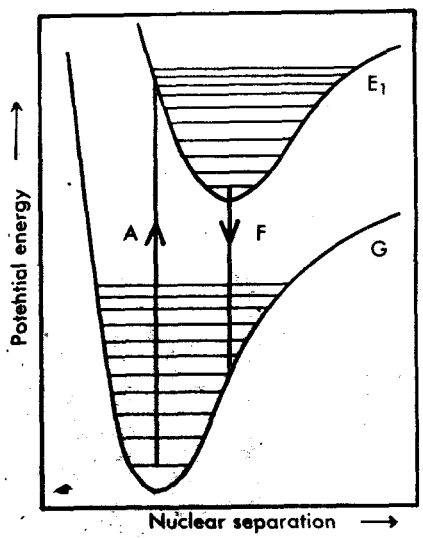


Fig. 1-2. Energy diagram of diatomic molecule **showing absorption of radiation, A,** and emission **of fluorescence, F. G is the** ground state level and **E represents the excited state.** (From Chadwick, C. S., and J. E. Fothergill. 1961. Fluorochromes and their conjugation with proteins. In R. C. Nairn [editor]. Fluorescent protein tracing. E. & S. Livingstone, Ltd., Edinburgh.)

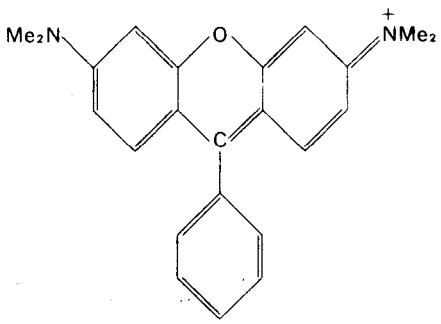
duces a characteristic white fluorescence. Many of the vitamins as well as hormones produce a fluorescence ranging from yellow to green or even bluish. Venoms, particularly those from the family Crotalidae (rattlesnakes), produce a pale yellow fluorescence when exposed to an ultraviolet light source. Intercellular material, notably connective tissue, produces a blue-white fluorescence (Price and Schwartz, 1956).

Knowledge of autofluorescence is also important in conjunction with various tracing studies, particularly when the substance in question is labeled with a fluorochrome that fluoresces at the same or nearly the same color as some of the autofluorescent material within the cell.

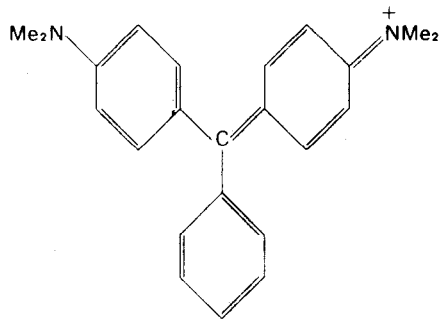
Metachromasia

When a stain such as toluidine blue, thionine, or cresyl violet is applied to a tissue section, much of the cytoplasm will stain the color of the dye, yet certain other areas within this same cell or material found in the intercellular region will stain a purple to red color. This change from the blue toward the red produces the metachromatic color. Most of the dyes that are capable of producing metachromasia contain the thiazine groupings. From a histologist's view, metachromasia occurs in substances that are half esters of sulfuric acid with a polymeric carbohydrate. This is evident in such substances as mucoproteins, chondrin (chondroitin sulfuric acid, which is the matrix of cartilage), and many other carbohydrate macromolecules.

For a long time the mechanism producing metachromasia was poorly understood. However, in 1945 Michaelis and Granick suggested after a series of experiments that metachromasia may be the result of the conversion of the monomeric form of the dye (which is blue or blue purple, depending on the particular dye used) to the polymeric form, which has a changed color, red or a shade of green. Sylevén (1954) suggested that metachromasia depends on several factors, one of the most important being the reaction of the dye to the substrate. The point of reaction is between the anionic groupings of the compound with the cationic group of the dye and between adjacent dye molecules aggregated to the substrate. It is assumed that polymeric formation is due to hydrogen bonding, probably mediated by water molecules between adjacent dye molecules in the aggregation. Furthermore, meta-



Rosamine



Malachite green

Fig. 1-3. Comparison of structure of rosamine (a fluorochrome) to that of malachite green, a nonfluorescing compound. Note absence of an oxygen bridge in malachite green. Probably the presence of the oxygen bridge is responsible for the fluorescing nature of rosamine. (From Chadwick, C. S., and J. E. Fothergill. 1961. Fluorochromes and their conjugation with proteins. In R. C. Nairn [editor]. Fluorescent protein tracing. E. & S. Livingstone, Ltd., Edinburgh.)

chromasia depends on a certain distance between available anionic surface charges. For example, in hyaluronic acid the anionic reaction groups are approximately 10 Å apart. This results in no metachromasia. On the other hand, when the densities are greater, that is, where a number of reactive groups are in the range of 4 Å, a stable metachromasia occurs.

Although it has been mentioned that metachromasia is specific for macromolecular carbohydrates, not all will give metachromasia. For example, polymers composed of hexosamine will not produce metachromasia. However, if the amino group is acetylated prior to treatment with a metachromatic dye, a positive reaction is produced.

Histochemistry

In a sense, histochemistry represents an extension of morphological staining techniques. Whereas the more routine techniques provide significant information regarding the morphology of the cell or tissue, little that is significant to the chemical implication involved can be noted. Histochemistry can be considered as the chemical identification and localization of various components in the cell, using techniques by which the end products are usually characterized by a colored precipitate or complex. In many reactions the final product can be quantitated spectrophotometrically.

The significance of histochemistry to cytological knowledge cannot be overemphasized. It is capable of changing a static field of morphology into a dynamic field closely related to biochemistry in which interpretation can be made on a structure-function basis.

Histochemistry, then, represents an interdisciplinary science that unites the various fields of chemistry, particularly organic chemistry and biochemistry, to the field of cellular or tissue morphology. Because of the dualistic nature of the field, the accuracy of localization is dependent on both the validity of the chemical reaction and on the ability of the reaction to maintain the architectural integrity of the tissue. Many basic reactions employed in biochemistry to identify specific compounds are useless in histochemistry because the reagents may be of such strength that they destroy the tissue. Often however, it is possible to so modify the reaction that the final result is not only valid for localization but does not unduly distort the tissue.

It is usually convenient to divide histochemistry into histochemistry per se and cytochemistry. Histochemistry consists of the chemistry of tissue. It is more general because it not only includes the identification of compounds within the cell and among various cells of the tissue but also the adjacent intercellular material and different adjacent tissues. On the other hand, cytochemistry is restricted to the chemistry of chemical changes observed within a particular cell or type of cell with little concern for the intercellular material.

As in the case of chemistry, the field of histochemistry can be subdivided into a number of specialized areas: (1) inorganic histochemistry, (2) protein histochemistry, (3) aldehyde histochemistry, (4) carbohydrate histochemistry, (5) lipid histochemistry, (6) nucleic acid histochemistry, and (7) the more functional field of enzyme histochemistry.

Inorganic histochemistry. Although numerous techniques for localization of metallic ions exist, only a few have been used with success. These include the localization of ion, calcium, and phosphate. Localization of other ions (for example, sodium and potassium), although techniques for identification and localization are available, involves a major problem produced by the interjection of the artifact of diffusion, which often can render the results useless. At best the results give merely regional distribution.

Several alternative methods are possible for distributional studies of inorganic ma-

terial. Microincineration or autoradiography can be used. Microincineration involves the ashing of tissue sections at a temperature of about 600° C. This method can provide information regarding general distribution of certain minerals such as calcium, magnesium, iron, and silicon (Pearse, 1960). After radioactive isotopes of such minerals have been given, autoradiography can also provide much information on the distribution of the various inorganic components. This can be achieved using either the light microscope or electron microscope. (See discussion of autoradiography.)

Protein histochemistry. A large number of the classical tests used in biochemistry cannot be used in general histochemical studies because of the violent effects on the tissue or because the color intensity may not be sufficient for interpretation at the microscopic level. In effect, histochemistry of proteins consists of tests that are selective for a specific amino acid or specific groups. Therefore these tests only detect parts of the protein molecule. One can assume that if a positive reaction occurs, the amino acid indicated is included in the protein structure rather than being an isolated amino acid in the tissue. This is based on the premise that during the processing of tissues, the free amino acids which may have been present in the tissue are removed.

At the present time only a few specific amino acids can be identified histochemically. By their relative proportions they also indicate acid, basic, or neutral proteins. They include arginine, lysine, histidine, tyrosine, and tryptophan. Other tests can be used for the localization of sulfhydryl (–SH–) and disulfide groups (–S–S–), which in effect, can be used to identify cystine and cysteine. Other tests are based on reactions to detect the free amino acid (NH_2) or carboxyl (COOH) and histone groupings.

Aldehyde histochemistry. Many of the histochemical tests used depend on the production of and detection of the aldehyde group. Much of the histochemistry of carbohydrates as well as the plasmal reaction of lipids and the Feulgen reaction for identification of DNA depend on the detection of freed aldehyde groups. Although some aldehyde can be considered to occur naturally in tissues, aldehydes are produced experimentally by selective oxidation. Usually the naturally occurring aldehyde is associated with fatty acids. The detection of aldehyde groups, whether they are free in tissues or produced by a selective oxidation process, is accomplished by Schiff's reagent.

Schiff's reagent. Although there are a number of reagents that could be used, the most common is Schiff's reagent, which is prepared from a basic fuchsin solution. This dye is treated with a sulfurous acid solution that converts the dark blue purple into a colorless solution. In this form it is commonly called *leucofuchsin* or *fuchsin sulfurous acid* solution.

When leucofuchsin reacts with available aldehyde groups, a red to red-purple color is developed. It is believed that two aldehyde groups react with one leucofuchsin molecule. Although many users of the reagent refer to the development of the color as a "recoloration" of the dye, actually it is more than a recoloration due to the oxidation of the reagent by the aldehyde group. The final color developed is different from that of the original dye, and it may be reasonable to assume that the reaction of the aldehyde has a chromogenic effect which is reflected by the red to red-purple color.

Carbohydrate histochemistry. In the preceding section the nature of the Schiff reaction in the detection of aldehyde groups was discussed. Going one step further, it can be stated that the field of carbohydrate histochemistry, including the true sugar polysaccharides as well as those formed by uronic acids and glucosamines and the conjugated polysaccharides, is dependent on Schiff's reaction. The procedure for the detection of carbohydrate is known as the

periodic acid–Schiff (PAS) reaction. In general, none of the carbohydrates will give a reaction by the direct use of Schiff's reagent. Instead, the aldehyde group must be produced by oxidation. In the PAS reaction periodic acid is used as the oxidant, which will break the C–C bonds where these are present as 1,2-glycol groups and convert them to dialdehyde (CHO–CHO). After oxidation the sections are treated with Schiff's reagent, which will develop a color where the aldehyde groups were formed (Fig. 1-4).

In addition to the PAS reaction a number of other carbohydrate tests are available. They include metachromasia as well as enzymatic action. The specific enzymes can be considered chemical reagents. The lack of staining characteristics after enzymatic action is indicative of the localization. Hyaluronidase, for example, is used for the detection of hyaluronic acid.

Lipid histochemistry. Because of the molecular structure of lipids, the histochemistry of this group is limited to the determination of classes of lipids rather than of the individual species. This is due to the similarity of both the chemical and the physical properties of the species within each class.

Probably the most interesting is known as the plasmal reaction. It was originally observed by Feulgen and Rossenbeck (Pearse, 1960). In this reaction a positive Schiff reaction was observed in the cytoplasm in sections that were not treated by acid hydrolysis. This indicates the presence of free aldehydes.

This reaction can be intensified by previous treatment with acid hydrolysis or by a short treatment with meruric chloride. This hydrolysis liberates the aldehyde group that can be detected by the Schiff reagent.

Nucleic acid histochemistry. Identification and localization are based on the following three components that compose nucleic acids: (1) the nitrogenous bases, (2) the deoxyribose sugar present in DNA, and

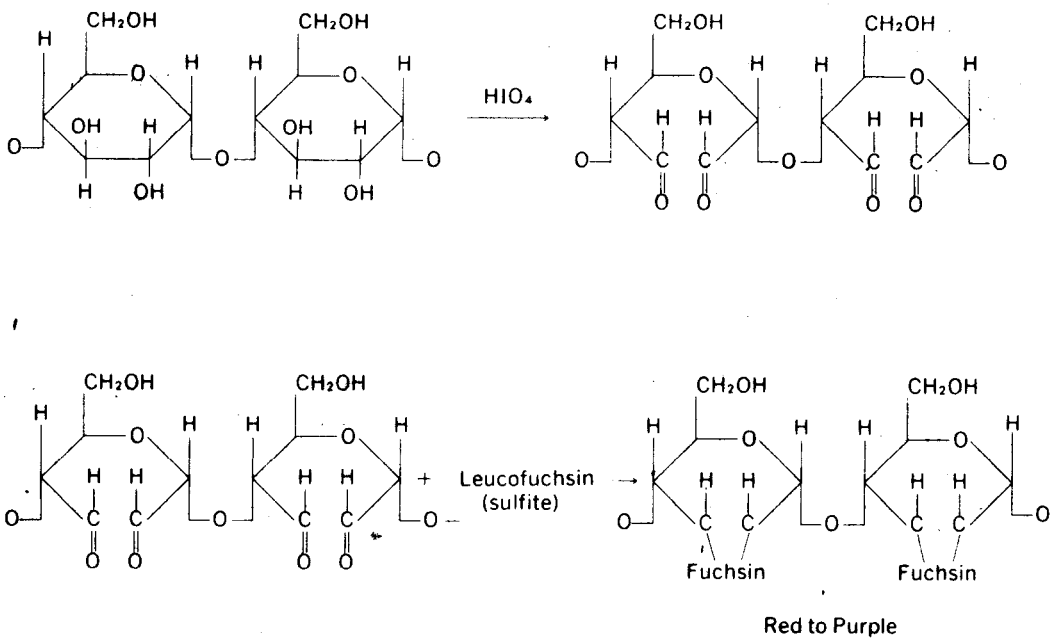


Fig. 1-4. Mechanism of periodic acid–Schiff reaction on carbohydrate.

(3) the phosphoric acid group associated with both DNA and RNA.

Nitrogenous bases. From the point of view of color identification, histochemical detection of purines and pyrimidines has been unsuccessful. However, the importance of the nitrogenous bases is that they will absorb ultraviolet light at 2,600 Å, and localization is made possible by microspectroscopy. However, the ultraviolet absorption technique will localize not only RNA but DNA as well.

Deoxyribose sugar. One of the basic differences between RNA and DNA is the sugar moiety present in the molecules. In DNA the sugar is deoxyribose, whereas in RNA the sugar is ribose. The presence of deoxyribose forms the basis of the Feulgen reaction, and so a positive reaction differentiates it from RNA, since the ribose sugar is not affected by the Feulgen reaction hydrolysis.

Feulgen reaction. As in the case of the PAS reaction, the basis of the Feulgen test is the detection of the presence of the aldehyde groups with Schiff's reagent. The Feulgen reaction can be divided into the following two stages:

1. The process of mild hydrolysis using a hydrochloric acid solution separates the purine bases from the deoxyribose sugar and exposes the aldehyde group of the latter. It is possible, however, by prolonged hydrolysis to separate the sugar group from the pyrimidine base as well.
2. The subsequent treatment of the aldehydes by Schiff's reagent produces a red-purple color.

This reaction can be studied photometrically to estimate the amount of DNA present. When used photometrically, the maximum absorption is at 550 nm.

Enzyme histochemistry. Enzyme histochemistry is not only the newest but the most expanding field of histochemistry. It represents a more functional approach to the study of cytology. Enzymatic studies at the cellular level can be of particular value in the field of pathology because they can explain the mechanism of action due to altered metabolism, but they can be equally important in helping to explain many of the reactions in cytological research.

The number of enzymes that can be visualized is large. To be sure, not all of those known can be detected and localized, but on the other hand, determining the distribution of those that are available can be used to obtain much of the information needed.

In general, the techniques used in enzyme studies are more exacting than those used in the other histochemical methods. In addition, the sources of error are more varied. Comparison of routine histochemical methods and enzyme studies reveals one striking difference. In the former the reagent will react with the constituent in the cell or intercellular material, and thus the final product is derived from the compound under study. In the latter the reagent is the substrate, which is acted on by the enzyme, and the product is the result of the enzymatic action. The resulting product must be highly insoluble when formed and should form a precipitate in situ so that the regions of the precipitate should be within the site of enzyme activity. In addition to its high insolubility in water, the precipitate should also have low solubility in lipids. Since many of the enzymes are situated on, in, or near membrane systems, solubility in the lipids would produce diffusion artifacts. The enzyme reaction must also obey the zero order of kinetics. That is to say, the rate of reaction must be independent of the substrate and related solely to the enzyme concentration.

Esterases. From the histochemical point of view esterases form a group of enzymes that catalyze or synthesize esters of carboxylic acids. The following diagram shows the reversible reaction:

RCOOR′	⇄	ROH	+ R′COOH
Carboxylic acid esters	Esterase	Alcohol	Carboxylic acid

A number of functions have been attributed to these enzymes. Meyers et al. (1957) suggest that esterase may play a role in protein metabolism because of its ability to hydrolyze amide and amino acid esters. It may also be related to the process of phagocytosis (Burstone, 1962). It is possible to divide the esterases into several groups: (1) aliesterases, which hydrolyze short-chain esters; (2) lipases, which hydrolyze fatty acids that have a long carbon chain; and (3) cholinesterases, which attack esters of choline.

Various histochemical methods are available for the demonstration of the different esterases. Among the most commonly used are indoxylacetate or the halogenated substituted form that reacts with an esterase to produce an indoxyl compound, which when followed by oxidation, produces an indigo dye. As in the case of phosphatases, a naphthol AS acetate can be used for a substrate, which when coupled with an azo dye, produces a colored precipitate. Gomori (1952) introduced the "Tween" method for detection of lipase. In this method a calcium salt soap of the fatty acid is produced, which on staining with, for example, Nile blue, produces a color indicating the localization of the enzyme.

Phosphatases. The phosphatases consist of a relatively large group of hydrolytic enzymes that catalyze the breakdown of phosphate esters to liberate phosphoric acid and an alcohol. Some of the phosphatases that can be demonstrated histochemically are listed in Table 1-1. Of particular importance are the phosphomonoesterases, which can be divided conveniently on the basis of their optimum pH. The phosphomonoesterases, broadly speaking, are nonspecific in that the enzyme will react with a number of different compounds or substrates. Within this nonspecific group of enzymes are the alkaline phosphatases (phosphomonoesterase I) and the acid phosphatases (phosphomonoesterase II). In addition to the nonspecific enzymes there are a number of enzymes that are substrate specific, including 5′-nucleotidase and glucose-6-phosphatase.

Visualization of phosphatase can be brought about by the metal salt method (Fig. 1-5) (Gomori, 1952) or by the use of one of the naphthol AS phosphates. In the former, either calcium or lead can be used, depending on the pH of the reaction. Calcium is used in an alkaline medium. The calcium combines with the phosphoric acid liberated to produce a calcium phosphate. The compound is converted successively to cobalt phosphate and finally to cobalt sulfide, which is a black precipitate. The lead method is similar. After the release of phosphates from the substrate a lead phosphate is formed, which is converted to lead sulfide

Table 1-1. Some common phosphatases that can be identified histochemically

Enzyme	Substrate
Phosphomonoesterase I (alkaline)	β-Glycerophosphate
Phosphomonoesterase II (acid)	β-Glycerophosphate
5′-Nucleotidase	Muscle adenylic acid
Adenosine triphosphatase	Adenosine triphosphate
Glucose-6-phosphatase	Glucose-6-phosphate
Glucose-1-phosphatase	Glucose-1-phosphate
Phosphoamidase	Phosphocreatine

for visualization. In the latter a number of naphthol AS phosphates are used. The phosphatases release the phosphate to produce highly insoluble naphthols, which are promptly coupled by an azo dye to produce a colored precipitate that can be visualized microscopically.

Dehydrogenases. Dehydrogenases consist of a group of enzymes that catalyze oxidation reactions by dehydrogenation. That is, these enzymes are capable of transferring the electrons from the substrate or proton donor to the oxidizing agent, which is the electron acceptor. Furthermore, these enzymes that catalyze dehydrogenation reactions are unable to utilize molecular oxygen directly as can the electron acceptor. Instead, there are a series of intermediate reactions in which the dehydrogenases transfer hydrogen to either NAD or NADP (di- or triphosphopyridine nucleotide) to produce the reduced state ($NAD \cdot H_2$ or $NADP \cdot H_2$). Furthermore, the reduced NAD and NADP are oxidized by a flavin nucleotide, FMN (flavin mononucleotide) or FAD (flavin adenine dinucleotide), which produce the oxidized NAD or NADP, but the flavoproteins are now reduced (Fig. 1-6). According to many investigators, the reduced flavoproteins under experimental conditions are capable of reducing dyes such as tetrazolium salts or methylene blue (Burstone, 1962). It is this point that forms the basis of the histochemical demonstration of a dehydrogenase enzyme. The reduced flavoprotein can be reoxidized by the dye, and the reduced dye formed will produce a colored insoluble formazan precipitate. However, there is some difference of opinion as to whether the reduced forms of FMN or FAD are capable of being oxidized directly by the dye.

A number of dehydrogenases can be localized histochemically. In Table 1-2 some of those that can be identified within cells are listed. By substituting the appropriate substrate and specific dehydrogenase in Table 1-2, the reaction as well as the mechanism of histochemical localization can be visualized.

Oxidases. Oxidases are enzymes that catalyze the transfer of electrons from a donor to oxygen. Many of the oxidases are metalloproteins and contain such metals as

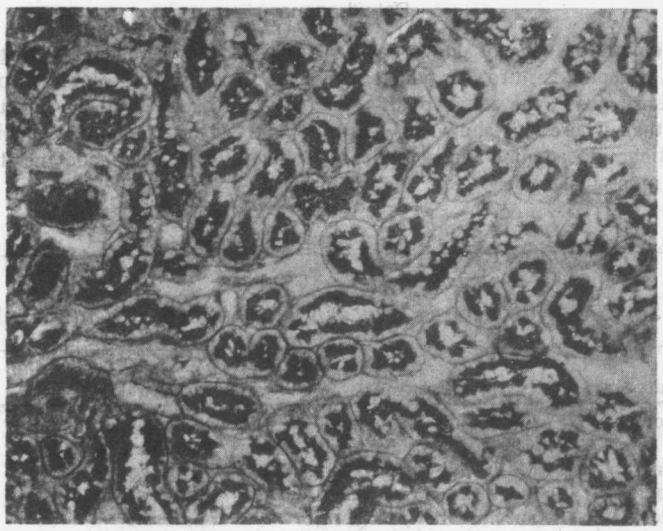


Fig. 1-5. Alkaline phosphatase activity in rat kidney. Note presence of enzyme activity along free surface of proximal convoluted tubules. Gomori's metal salt method.