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Enzyme Histochemistry

A Laboratory Manual

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With 20 Figures

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Preface

During recent years enzyme histochemical reactions have increasingly been considered as important, the reason being that enzyme histochemistry is now a well-established link between morphology and biochemistry. The development of numerous new methods and in particular the improvement of existing techniques contributed to the expansion of enzyme histochemical reactions. Today, the use of these methods allows detailed insight into molecular processes of single cells and their constituents.

The selection of a suitable method for enzyme histochemical investigations needs thorough knowledge and critical evaluation of the reactions described for the histochemical demonstration of enzymes and introduced in laboratory practice. Often, it is difficult for scientists primarily concerned with the application of methods and for laboratory assistants to comment on the value of an enzyme histochemical reaction. Our book will serve as a guide in this respect. It contains the most important histochemical methods for the localization of enzymes, all of which were checked by the authors themselves. These methods were often modified and frequently used for numerous different investigations of healthy and diseased organs in basic research and in routine practice.

Compared with the German edition, the English version is enlarged and improved. Especially, methods for hematologic diagnosis and new reactions, e.g., for the demonstration of peptidases, were included in the book. However, as stated in the German edition, methods with which no experience exists or which, in our hands, deliver unsatisfactory results are mentioned but not recommended for practical use. Therefore, this manual represents a critical collection of enzyme histochemical methods for practical purposes in the histochemical laboratory. We pay attention to theoretical aspects in so far as they are necessary for the understanding of problems which may arise in practical histochemistry and of biochemical processes and connections.

We are thankful to our co-workers who have supported us since we began work in the field of enzyme histochemistry. It is impossible to name all of them personally. Furthermore, we thank the staff of Springer-Verlag, Heidelberg who gave us much helpful advice during the preparation of the manuscript.

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Contents

A. Introduction

Enzymes are found in all plant and animal organisms; they can be studied biochemically as well as histochemically. The advantage of histochemical methods is that detection takes place in situ, which allows the localization of enzymes in normal and pathologically altered organs, tissues, and cells.

Chemically enzymes are macromolecules, the most substantial component of which consists of proteins. They catalyze metabolic reactions; their capacity for catalysis is connected with the presence of active sites that can convert a substrate. Most histochemical techniques for the detection of enzymes are based on this property. Another approach is based on the proteinaceous nature of enzymes. This embraces the immunohistochemical methods, which are not treated in this book.

In the histochemical detection of enzymes it is important to obtain and visualize a product of the enzymatic reaction at the site of occurrence of the enzyme. The structural integrity of tissues and cells must be sufficiently well preserved to allow correct localization and evaluation of the enzymatic activity.

B. General Considerations

I. Conditions for the Histochemical Demonstration of Enzymes

In every histochemical investigation of enzymes the following requirements should be considered and fulfilled as completely as possible:

1) The preparation of tissues and sections should not influence the distribution and activity of the enzyme.
2) The substrate and auxiliary reagents should penetrate all cells and their components with equal speed.
3) The substrate should be split if possible by one enzyme only.
4) The auxiliary reagents should neither interfere with the enzymatic reaction nor hinder the penetration of the substrate.
5) The product of the enzymatic reaction should be captured by the auxiliary reagent very quickly; this should occur independently on the cell milieu.
6) The final product should precipitate immediately, i.e., it should be practically insoluble in aqueous solutions and in lipids. Moreover, it should be amorphous (or at least microcrystalline) and stable.
7) The substances participating in the reaction should not be bound or adsorbed onto other structures than the enzyme-reactive sites.

Usually it is very difficult to meet all these requirements. Every histochemical enzyme reaction is therefore based on a compromise.

II. Terminology and Classification of Enzymes

Enzymes are distinguished by the suffix "-ase" and, as a rule, designated according to the substrate they split. When a different substrate is used in histochemical determinations than in biochemical tests, and it has not yet been proved that both are split by the same enzyme, it is recommended that the enzyme determined in situ be designated according to the special substrate used, e.g., that an enzyme that splits L-leucyl-2-naphthylamide be referred to as a "leucylnaphthylamidase."

In the meantime, biochemistry has evolved a systematic and rational nomenclature of enzymes (Enzyme Nomenclature, 1972; BARMAN, 1969 a, b, 1974). Accordingly, an enzyme is characterized by four numerals. The first numeral indicates to which of the six classes the enzyme belongs. Class 1 enzymes are the oxidoreductases, class 2 the transferases, class 3 the hydrolases, class 4 the lyases, class 5 the isomerases, and class 6 the ligases (synthetases). The second numeral indicates the subclass in each case. The third numeral indicates the appropriate sub-subclass, and the fourth numeral the ordinal number of the enzyme within the sub-subclass in question. Thus, for example, alkaline phosphatase is characterized by the number 3.1.3.1. This means that it is one of the hydrolases, which split ester bonds of phosphoric acid monoesters.

III. Principles of Reactions in Histochemical Methods for the Detection of Enzymes

1. Precipitation Reactions

a. Precipitation Reactions with Metallic Cations (Gomori-Type)

The reactions comprise several steps, which are carried out in different media:

1) Splitting of the substrate (primary or splitting reaction). Natural substances are usually used.

2) Precipitation reaction (secondary reaction).
The splitting and precipitation reactions take place in the same medium. For the precipitation of the product of the enzymatic reaction Ca^{2+}, Pb^{2+}, Cu^{2+}, or Ba^{2+} ions are added. As a rule, the precipitate is not visible under an ordinary light microscope, or only barely so; it is detectable with a phase-contrast or polarizing microscope.

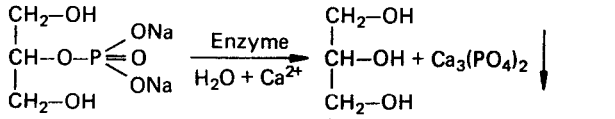
3) Visualization (tertiary or transformation reactions).
A colored reaction product easily recognizable under the light microscope can only be obtained if the precipitation reaction is followed by one or more further reactions.

Topically correct precipitation depends on a sufficient concentration of metallic cations in the incubation medium. On the other hand, the metal cations inhibit enzymatic reactions and certain cellular structures are metallophilic. Finally, the confusion of colored reaction products with genuine pigments or preformed calcareous deposits is a critical factor.

The principle of the classic Gomori-type reaction is the basis for the detection of phosphatases, sulfatases, and cholinesterases.

Examples: Alkaline Phosphatase

1) Splitting and precipitation reaction

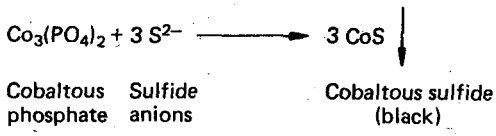


Sodium salt of 2–glycerophosphate — Glycerin — Calcium phosphate (white)

2) First transformation reaction (fresh medium)

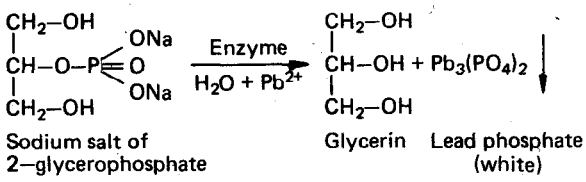
Calcium phosphate — Cobalt cations — Cobaltous phosphate

3) Second transformation reaction (fresh medium)

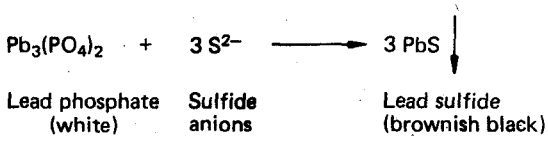


Acid Phosphatase

1) Splitting and precipitation reaction

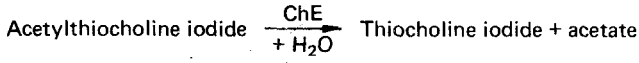


2) Transformation reaction (fresh medium)



Cholinesterase (ChE; reaction after KARNOVSKY and ROOTS, 1964): In this reaction Fe^{3+} in the form of potassium ferricyanide (potassium hexacyanoferrate(III)) is added as a heavy metal cation or precipitating reagent. Certainly this means the reaction is no longer a classic Gomori-type one. Potassium ferricyanide is reduced to potassium ferrocyanide by thiocholine iodide released from the substrate by enzymatic activity. The potassium ferrocyanide is then precipitated by Cu^{2+} ions as brown $Cu_2Fe(CN)_6 \cdot 7\ H_2O$ (Hatchett brown). All these reactions take place in the same incubation medium.

1) Primary reaction



2) Secondary reaction (transformation reaction)

Potassium ferricyanide (potassium hexacyanoferrate (III)) —Thiocholine iodide→ Potassium ferrocyanide (potassium hexacyanoferrate (II); still diffusible)

3) Tertiary reaction (precipitation reaction)

Potassium ferrocyanide + Cu^{2+} ⟶ $Cu_2Fe(CN)_6 \cdot 7\ H_2O$ ↓

(potassium hexacyanoferrate (II)) . Hatchett brown

This reaction principle is also used in the demonstration of other enzymes, especially in ultracytochemistry. For the visualization of acid phosphatase or nonspecific esterase activity potassium ferricyanide is reduced by thio compounds that are released from the substrate, e.g., 2-naphthylthiolphosphate or benzoylphenylthiolacetate (HANKER et al., 1972). For the demonstration of oxidoreductases potassium ferricyanide is used instead of tetrazolium salts (see tetrazolium salt methods) as an artificial acceptor of electrons (HANKER et al., 1973). The formation of Hatchett brown may also be used for the detection of enzymes by methods using indoxyl substrates, in which potassium ferricyanide acts as an oxidation agent (PUGH, 1972; see p. 12). However, in this case a higher incidence of diffusion artifacts must be accepted than in the reaction for cholinesterases.

All reactions in which microscopically visible Hatchett brown is formed may either be evaluated directly or be followed by further reaction steps. Either osmium tetroxide (OsO_4) is changed to osmium black by thiocarbazide bound to Hatchett brown, or Hatchett brown acts as a metallic catalyst in the oxidative polymerization of diaminobenzidine. This polymer reacts with OsO_4 in a sequential reaction; the end product is again osmium black, which is insoluble in water and lipids.

b. Simultaneous Azo-Coupling (Simultaneous Coupling)

This involves two-step reactions:

1) Splitting of the substrate (primary or enzyme-splitting reaction). Naphthol derivatives act as substrates; the ones most often used are 1- or 2-naphthol and naphthol AS compounds (Naphtholanilidsäure, AS Anilidsäure). Naphthylamine, indoxyl, and indolylamine derivatives can also be used as substrates. The action of enzymes leads to the formation of naphthol, naphthol AS, naphthylamine, indoxyl, or indolylamine (primary reaction product).
2) Coupling and precipitation reaction (secondary reaction). A diazonium salt added as a coupling reagent reacts with the primary reaction product and the formation of a water-insoluble (but somewhat fat-soluble) azo dye results.

The principle of simultaneous azo-coupling can be used for the detection of phosphatases, glycosidases, carboxylic esterases, and peptidases.

Substrates: Unsubstituted and substituted naphthol, naphthylamine, indoxyl and indolylamine derivatives are employed. In the case of the indoxyl and indolylamine substrates the reaction is designated an azoindoxyl procedure. The substituted naphthol derivatives most frequently used are naphthol AS and 6-bromonaphthol compounds. The derivatives of naphthol are used for the detection of phosphatases, glycosidases, and esterases. Naphthylamine and indolylamine derivatives are used for the detection of peptidases; indoxyl substrates are employed for the detection of esterases, glycosidases, and phosphatases. In general, the derivatives of simple naphthol are hydrolysed faster than naphthol AS compounds. Of the unsubstituted naphthol substrates, the derivatives of 1-naphthol are more suitable for histochemical purposes than those of 2-naphthol (GOSSRAU, 1976 b). Many naphthol AS compounds are commercially available; however, only a few are useful for the practical histochemistry of enzymes, namely naphthol AS-BI,

naphthol AS-D, naphthol AS-MX, and naphthol AS-TR substrates (LOJDA et al., 1964, 1967). Of the naphthylamine derivatives, those of 4-methoxy-2-naphthylamines are preferable to unsubstituted 1- or 2-naphthylamines, due to their higher coupling velocity; the rate of cleavage is generally the same (GOSSRAU, 1976a, 1978 d; LOJDA, 1977 a, b, 1978). In the group of unsubstituted naphthylamine derivatives the 1-naphthylamine substrates are superior (LOJDA, 1975 b). Among the indoxyl and indolylamine derivatives the splitting rate is higher in the case of substituted compounds; 5-bromo derivatives are very suitable in azo-indoxyl reactions.

Coupling agents (diazonium salts): The numerous commercially available stable diazonium salts are designated "Fast" or "Echt", followed by certain letters, sometimes in combination with numerals related to the chemical structure of the fast salt. In the case of Fast Blue B (the English designation is used internationally), "Fast" means a stable diazonium salt and "Blue" the color produced after coupling with 2-naphthol, while "B" refers to the chemical formula. Fast Blue B is the stable diazonium salt of *o*-dianisidine.

Diazonium salts important in practice: Fast Blue B = tetrazotized *o*-dianisidine, Fast Blue BB = diazotized 4-benzoylamino-2,5-diethoxyaniline, Fast Blue RR = diazotized 4-benzoylamino-2,5-dimethoxyaniline, Fast Red TR = diazotized 5-chloro-2-toluidine, Fast Garnet GBC (Echtgranatsalz GBC) = diazotized 4-amino-1,3-dimethylazobenzene, and Fast Blue VB =diazotized 4-methoxy-diphenylamine.

Commercial stable diazonium salts are normally supplied as diazonium chloride-zinc chloride double salts, fluoborates, or naphthalene disulfonates; only a small number of diazonium salts exist as pure chlorides. Moreover, aluminium, sodium, magnesium, and zinc salts are present as stabilizers in many diazonium salts. Most batches of diazonium salts contain only about 20% of substance capable of coupling.

Hexazonium-*p*-rosaniline and hexazotized new fuchsine are the diazonium salts of choice for most azo-coupling reactions in enzyme histochemistry. They are not supplied as stable salts and must always be prepared shortly before use.

Criteria in work with diazonium salts: The most important factors to be checked are the stability of the diazonium salts in the incubation medium, the velocity of coupling, the rate of inhibition, and the particle size of the azo dyes formed.

The *stability* of diazonium salts in the incubation medium depends on pH, temperature, concentration, and the type of diazonium salt. With rising pH a salt becomes less stable, i.e., its degradation becomes faster. The degradation products are responsible for most of the background staining of the sections; this is also caused, to a lesser degree, by the diazonium reaction with certain amino acids of tissue proteins. The instability of diazonium salts at alkaline pH and 37°C may make a change of incubation medium necessary when longer incubation times are required. Under identical conditions Fast Red TR is more stable than Fast Blue BB and RR; Fast Blue B and Fast Garnet GBC are the least stable of the diazonium salts mentioned.

The *coupling velocity* is of decisive importance for correct localization of the enzyme. It is influenced mainly by the pH and by the coupling partner (primary reaction product). If substituted and unsubstituted naphthols are used the coupling speed increases with rising pH, so that there are better conditions for a true-to-site localization in the detection of alkaline phosphatase activity than in the demonstration of neutral glycosidases (glucoamylase, lactase); conditions for correct localization are worst in the case of acid hydrolases (acid phosphatase, acid glycosidases). The naphthylamines couple more slowly than naphthols in the pH range 6-8.

Often a compromise must be made, i.e., a pH has to be selected that although not optimal for the enzyme activity, would allow a coupling reaction quick enough to make exact localization of the azo dye possible and at the same time leave the enzyme sufficiently active.

Rate of inhibition: Individual enzymes are inhibited by diazonium salts to varying degrees. The inhibition of a given enzyme is caused by the diazonium salt itself, by the stabilizers it contains (e.g., Al^{3+}, Mg^{2+}, Zn^{2+}), and by its concentration. The rate of inhibition also depends on the pH at which the azo-coupling reaction takes place. In view of the inhibitory effect of diazonium salts, the smallest possible amounts should be used in simultaneous coupling reactions. On the other hand, good localization of reaction products requires sufficiently high speeds of coupling, which depend on a certain minimum amount of diazonium salt. A good compromise is 1 mg/ml for stable diazonium salts (0.5 mg/ml is enough for very high quality diazonium salts containing a high portion of effective coupler); 0.03-0.09 ml/1 ml for hexazonium-*p*-rosaniline (naphthol, naphthol AS and naphthylamine compounds); 0.002-0.025 ml/1 ml for indoxyl substrates and for hexazotized new fuchsine 0.008-0.08 ml/1 ml (naphthol and naphthol AS derivatives), 0.015-0.03 ml/1 ml (naphthylamine substrates) and 0.005-0.025 ml/1 ml (indoxyl derivatives).

Particle size: The coupling partners and the pretreatment of the tissue samples determine the particle size. In histochemical studies on paraffin, freeze-substituted, or freeze-dried cryostat sections most azo dyes appear amorphous or microcrystalline, whereas in cryostat or frozen sections of aldehyde-fixed or fresh material without lipid extraction the dyes usually appear granular. In cryostat or frozen sections only hexazonium-*p*-rosaniline and hexazotized new fuchsine, and of the stable diazonium salts only Fast Blue B, form the amorphous or microcrystalline reaction products that are necessary for the assessment of the intracellular localization of enzymes. When fresh cryostat sections are treated with a cold acetone-chloroform mixture the size of azo dye particles appears smaller than in untreated sections.