Methods of Detection of Specific Enzymes

This part is central for the book. It contains detailed descriptions and outlines of more than 900 enzyme-specific zymogram techniques suitable for visualization on electrophoretic gels of more than 400 different enzymes. The information presented herein is traditionally arranged around the “enzyme sheet,” which includes basic information required for the detection of a particular enzyme. At the beginning of this part, additional information of general relevance to the enzyme sheet of a given enzyme is given. It includes a description of the structure of an enzyme sheet, general considerations, and comments and recommendations concerning application of zymogram techniques, as well as resource-saving strategies, troubleshooting, and safety measures. Abbreviations commonly used in the enzyme sheets are also given.

SECTION 1

THE STRUCTURE OF ENZYME SHEETS

The enzymes considered in this part are presented in numerical order according to the enzyme code (EC) numbers recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) in 1992. Each enzyme sheet is presented in a set format and includes information on enzyme reaction, enzyme source, subunit structure of enzyme molecules, specific zymogram methods (with four types of information given for each method: visualization scheme, staining solution, procedure, and notes), additional methods, general notes, and references.

As a rule, the recommended enzyme name is given in the enzyme sheet headline, while additional enzyme names are presented for cross-reference purposes in the section “Other Names.” The enzyme symbols used are the same as the enzyme abbreviations most commonly encountered in the literature. Some symbols (while absent from the literature) are constructed as uppercase abbreviations in accordance with specific recommendations. Thus, the enzyme sheet heading includes the EC number and the full and abbreviated names of the enzyme, for example: 1.1.1.1 — Alcohol Dehydrogenase; ADH. An alphabetical list of all the enzymes considered is given for convenience in Appendix B.

The enzyme reaction adapted from the enzyme list published by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology is given in each enzyme sheet under the subheading “Reaction.” Groups of living organisms that are sources of the specific enzyme are listed in the section “Enzyme Source” to address more directly the application of specific zymogram methods to certain groups of organisms. This information was selected from Dixon and Webb’s book, Enzymes, current literature, current reference books, catalogs of chemical companies, and the Science Citation Index database of the Institute for Scientific Information (Philadelphia).

The knowledge on the subunit structure of enzymatic molecules is of fundamental importance for an adequate interpretation of banding patterns developed on zymograms by methods given in the book. Information on the subunit structure, if available, is given in the section “Subunit Structure.” An enzyme is indicated as a monomer if its native molecules are represented by single polypeptides. The subunit structures of homomeric enzyme molecules (i.e., those consisting of several identical subunits) are indicated as dimer, trimer, tetramer, etc., and those of heteromeric enzyme molecules as heterodimer, heterotrimer, heterotetramer, etc. The subunit composition of heteromeric enzyme molecules, if available, is indicated in parentheses (e.g., heterodimer (αβ), heterotetramer (αββγ), heterotetramer (2α2β)), etc.) or described in more detail in the section “General Notes.” Some authors believe that the subunit structure of most enzymes is highly conserved throughout the organismal evolution so that the subunit structure known for vertebrates may serve as a reliable indication for all other animal groups and even plants. Although this is true for many enzymes, some enzymes demonstrate different subunit structures in different higher taxa and even within them. This suggests that information on subunit structure should be as complete as possible to make the interpretation of banding patterns detected on zymograms adequate and reliable. Higher taxa in which the subunit structure of the enzyme is determined are listed in parentheses. If the subunit structure of an enzyme varies within a higher taxon (a situation common in bacteria), more detailed taxonomic information may be given. Conventional biochemical methods necessary to detect the subunit structure of enzyme molecules require purified enzyme preparations that are laborious and time consuming, and therefore not suitable for wide phylogenetic surveys. The combination of enzyme electrophoresis and the zymogram technique is of great advantage in this respect because it allows the determination of the subunit structure of enzyme molecules quickly and comparatively easily, using unpurified enzyme preparations (e.g., tissue homogenates). This is due to a well-defined relationship between electrophoretic patterns of enzyme activity bands revealed on zymograms and the subunit structure of native enzyme molecules. The subunit numbers can be inferred from allozyme patterns observed in heterozygotes as well as from isozyme patterns detected in interspecific F₁ hybrids, somatic cell hybrids, and in vitro hybridization experiments. Such information is now available for about one third of enzymes included in the book (author’s unpublished data). For many of these enzymes the subunit structure is determined in a wide...
Detection of Enzymes on Electrophoretic Gels

phylogenetic range of living organisms (e.g., see 1.1.1.37 — MDH; 1.1.1.42 — IDH; 1.1.1.44 — PGD; 2.6.1.1 — GOT; 5.3.1.9 — GPI; 5.4.2.2 — PGM). The “Subunit Structure” section contains two types of information. The first type includes data obtained by conventional methods (mostly by combined native PAG and SDS-PAG electrophoresis of purified enzyme preparations). These data are obtained from the Science Citation Index database (Web-of-Science) of the Institute for Scientific Information (Philadelphia). They are marked by the superscript # (i.e., monomer#, dimer#, trimer#, etc.). The second type includes data inferred from banding patterns detected on zymograms. This information is given without any marking (i.e., monomer, dimer, trimer, etc.).

More than one principally different zymogram technique is given for many enzymes. Information on each separate technique is given in the section “Method,” which is subdivided into four subsections: “Visualization Scheme,” “Staining Solution,” “Procedure,” and “Notes.”

The diagram of the reaction sequence involved in the enzyme detection is schematically pictured in the subsection “Visualization Scheme.” A general principle was used to construct such diagrams for the great majority of the enzyme detection methods described in this book. According to this principle, arrows indicate the direction of the reactions leading to visualization of the enzyme activity bands on the electrophoretic gels. All the participants of the enzyme detection reactions are given. Reagents included in the staining solution are given in bold type. All enzyme names are abbreviated, with the abbreviated names of the linking enzymes deciphered in the recipe of the staining solution. The abbreviated name of the enzyme being detected is enclosed in a box. The final product that enables the enzyme to be visualized is indicated by UV when registered in ultraviolet light or VIS when registered in daylight; for those compounds that change color during the reaction, VIS ′ indicates the initial color and VIS indicates the color after the change. When the visualization procedure is a compound process that should be carried out sequentially step-by-step, different stages of the procedure are indicated on the reaction diagram. In some cases (e.g., when the scheme of an autoradiographic procedure is given) different stages depicted on the diagram are supplied with additional information for convenience (e.g., Stage 1: Enzyme reaction; Stage 2: Washing the gel; etc).

The subsection “Staining Solution” ("Reaction Mixture" and "Indicator Agar" for autoradiographic and bioautographic methods, respectively) gives the recipe for the reaction mixture used to visualize areas occupied by the enzyme on the electrophoretic gel. The reference for the given recipe is also presented. Efforts have been made to obtain the recipes from the original articles in which they were first published. This goal, however, was not always achieved because many of the original recipes were modified throughout the years and the accumulated changes were not always well documented. The recipe may include several subrecipes, designated A, B, C, etc., when the compound-visualization procedure should be carried out in a step-by-step fashion or when the preparation of the staining solution should be carried out by successive mixing of solutions prepared separately according to subrecipes. As a rule, the recipes are given in the form in which they are presented in the cited paper. As a consequence, in some recipes the involved reagents are given in absolute quantities, while in others they are given in concentrations. Each of the two modes of presentation of reagents in a recipe has its own advantages and disadvantages; therefore, no attempt has been made for overall standardization of the recipe presentation.

The subsection “Procedure” contains a detailed description of sequential procedures leading to the development of enzyme activity bands on an electrophoretic gel and thus represents a peculiar “know-how” of the method. It may be subdivided when a compound procedure of the enzyme visualization is described. Information on the mode of documentation of results obtained and some peculiarities of preservation of stained gels is also given there.

Diverse information that is of value for more successful application of the method and more adequate interpretation of obtained results is given under the subheadline “Notes.” This information (if any) includes remarks concerning possible artifacts generated by the method and recommendations on control gel stainings, when they are needed. It also includes recommendations concerning possible counterstaining procedures used to make stained bands more contrasting and clearly visible, highlights some important details of the mechanism involved in the enzyme detection system, etc.

The section “Other Methods” initially included those methods for which the author had information concerning only general principles of the enzyme visualization. However, during the writing of the book this section was filled with methods that seemed supplementary or not widely used. For example, such detection methods as bioautography, two-dimensional spectroscopy, and immunoblotting were referred to as “Other Methods” when more simple and practical or commonly used zymogram methods were available for the enzyme under consideration. Methods that seemed adequate but had not been used so far to detect the enzyme under consideration were also given under “Other Methods,” as recommended for application. It is not excluded for some enzymes that such methods may prove even more useful than the methods given in the “Method” section.

“General Notes” are given after the description of all the main zymogram methods developed for the enzyme under consideration. These notes give additional information that is of value for all the methods described for the enzyme. These are remarks on the enzyme substrate specificity, specific inhibitors and activators, comparative analysis of different detection methods, etc.

The list of “References” is given for each enzyme at the end of the enzyme sheet. The original papers are included in the list when possible to indicate the priority in development of the enzyme detection methods. In some cases where there are many references, those that contain reviews are preferred.
Methods of Detection of Specific Enzymes

REFERENCES


SECTION 2
GENERAL CONSIDERATIONS, COMMENTS, AND RECOMMENDATIONS

THE CHOICE OF SUPPORT MEDIUM FOR ENZYME ELECTROPHORESIS AND DETECTION

The principles of detection of enzymes on electrophoretic gels are not dependent upon the type of support medium used for enzyme separation. However, in practice, the adequate choice of support medium is of great importance. This is because:

1. The quality of zymograms obtained via enzyme detection methods depends on characteristics that differ significantly in different support media.
2. A certain support medium may be preferable when a certain biological object is used as the source of the enzyme.
3. The choice of a certain support medium may depend on the question to be answered and the resources available to the researcher.
4. A number of zymographic methods are adapted or are recommended to be preferably applied to a certain support medium.

At present the most popular support media are starch, cellulose acetate, and polyacrylamide. Many of the characteristics that should be taken into account during the choice of support medium have been discussed by many authors. The most important of them are considered below.

Cellulose Acetate Gel

Cellulose acetate gel requires only 0.5 to 2 µl per sample per enzyme run, and thus is the medium of preference when many enzymes must be detected from single small samples. The use of cellulose acetate may be highly desirable when small samples are more easily obtained than large ones (e.g., such a situation is common for isozyme studies of cell cultures or cultures of microorganisms). It is also preferable when enzyme stains containing very expensive ingredients should be used. This is because the 30 × 15 cm cellulose acetate sheets usually require not more than 3 ml of the staining solution. Cellulose acetate gels are commercially available and are ready for loading with samples after 10 min of soaking in an appropriate electrophoretic buffer. Only 1 h is usually needed to run cellulose acetate gels using relatively low run voltages. This allows, if necessary, the electrophoretic separation of enzymes to be carried out at room temperature without special cooling devices. Enzyme activity bands develop on cellulose acetate gels quickly because of the high porosity of the gel matrix and since the ingredients of the staining solution easily diffuse into the gel. This advantage is especially valuable when the procedure for the enzyme detection involves an exogenous linking enzyme(s) of high molecular weight(s). Electrophorized cellulose acetate gels can be frozen before or after the completion of the staining and thus can be available for further reference. Finally, cellulose acetate gels are durable and flexible, allowing the stained gel to be handled without unnecessary caution.

On the other hand, cellulose acetate gels cannot be sliced into a number of thin gels in the fashion of starch gel blocks. They are more expensive than equivalent starch or polyacrylamide gels, not translucent, and so cannot be quantified by densitometric methods without additional treatment. Cellulose acetate gels display reduced stain intensity for isozymes with low activity because only small volumes of samples can be applied to the gel. This support medium does not have the ability to cause a molecular sieving effect because of its coarse pore structure. So it separates proteins primarily on charge, with little or no separation on size. Finally, electroendosmosis does occur to an appreciable extent with just this support medium. In practice, however, at least some of these disadvantages are not critical. Indeed, electroendosmosis usually does not affect the relative mobility of allozymes and isozymes. Again, it may be reduced...
by the use of relatively more concentrated electrophoretic buffers. The absence of the molecular sieving effect does not play any important role in separation of allozymes, which usually do not differ markedly in molecular weight or shape. Gel slicing may offer no advantage in the peculiar situations that commonly occur during biochemical systematic studies. For example, during interspecific comparisons, each compared locus may require specimens from different species to be applied to a gel in a locus-specific order, placing side by side just those allozymes that need to be tested for identity or difference. As to the relatively high cost of the cellulose acetate gels, it should be pointed out that equivalent starch or polyacrylamide gels are actually more costly when labor costs are included. If densitometric scanning is desired, stained cellulose acetate gels can be cleared by washing them in 5% acetic acid, treating with 95% ethanol for 1 min, and immersing in 10% acetic acid and 90% ethanol mixture for 5 min. Before scanning, the gel should be dried and heated on a glass plate at 60 to 70°C for 20 min.

It should also be kept in mind that the use of cellulose acetate strips (i.e., the nongel form) does not give as good resolution of allozymes and isozymes as cellulose acetate gels do. Cellulose acetate gels are produced by a number of manufacturers.

The excellent handbook by Richardson et al. contains comprehensive information on the application of cellulose acetate gels for isozyme and allozyme analysis and is recommended for more detailed consultation.

**Polyacrylamide Gel**

This support medium is formed by the vinyl polymerization of acrylamide monomers and cross-linking of the formed long polyacrylamide chains by the bifunctional co-monomer \(N,N'\)-methylen-bis-acrylamide in anaerobic conditions in the presence of such catalysts as ammonium persulfate and \(N,N,N',N'-\)tetramethylethylendiamine, or 3-dimethylaminopropionitrile. The main advantages of polyacrylamide gels are as follows:

1. The composition of the gel can be modified in a controlled way to achieve the best separation of the isozymes under question due to the optimal molecular sieving effect.
2. The gel matrix is highly homogeneous.
3. The gel is clear and can be directly subjected to quantification of enzyme activity bands by densitometry.
4. The results obtained with polyacrylamide gels are highly reproducible.
5. The use of this support medium allows detection of enzymes after electrophoresis of very dilute samples (e.g., some biological fluids, extracts of algal tissues, etc.).
6. The denaturing SDS–polyacrylamide gel system is the only electrophoretic medium suitable for analysis of many nonwater-soluble monomeric enzymes that can be renatured after appropriate treatments.
7. The sharpest protein bands are obtained with this support medium.
8. The polyacrylamide gels are rigid and thus are convenient for handling.

It is commonly acknowledged at present that the polyacrylamide gel is the best medium for separation of different classes of nonenzymatic proteins. This is, however, not always true for enzymatic proteins because of at least several pronounced disadvantages of this gel toward electrophoretic analysis of enzymes.

The first and the most critical disadvantage is the formation of nongenetic secondary isozymes as a result of the action of residual nonreacted persulfate, an oxidizing agent, which can cause structural modifications of enzyme molecules or the loss of their catalytic activity. Polyacrylamide gels always contain a small residual fraction of unpolymerized acrylamide monomers, which also can react with enzymatic molecules. This drawback may be eliminated by preelectrophoresis treatment, but only if continuous electrophoretic buffer systems are used. When the use of discontinuous buffer systems is needed, the polymerized gel should be soaked for a period of days in several changes of an appropriate gel buffer. This diminishes the value of the polyacrylamide gel in large-scale electrophoretic analysis of enzymes.

The next disadvantage of the polyacrylamide gel is the almost total impossibility to slice gel blocks into thin gel plates. This considerably limits the multilocus analysis from one electrophoretic run.

When vertical polyacrylamide gels are used, only isozymes moving toward one electrode (usually anode) are detected. Hence, two separate runs are needed to detect both anodally and cathodally moving isozymes. This problem can be avoided by the use of horizontal gel slabs, which allows the placing of samples anywhere in the slab. Positioning samples in the middle of the gel causes isozymes migrating toward either or both electrodes to be observed on the same gel. The use of horizontal gel blocks, however, does not allow exploitation of the most important advantage of vertical polyacrylamide gels, i.e., its value for analysis of very dilute samples with low enzyme concentration.

A problem with detection of some enzymes on polyacrylamide gels can occur when visualization mechanisms involve linking enzymes of large molecular weight. This is because of the slow diffusion of such enzymes into the polyacrylamide gel matrix. Indeed, most separations of enzymes are carried out with 5 to 12% polyacrylamide gels, which optimally separate proteins with a molecular weight range of 20,000 to 150,000. So, diffusion of such routine linking enzymes as, for example, xanthine oxidase from milk (mol wt 290,000) or glutamate dehydrogenase from liver (mol wt 1,000,000) into commonly used 7.5% gels will be very slow, if at all.

Finally, both acrylamide and bis-acrylamide are highly toxic, and even very dilute solutions of these monomers can cause skin irritation and disturbance of the central nervous system. Polymerized gel, however, is relatively nontoxic and can be handled safely.
Methods of Detection of Specific Enzymes

Starch Gel

At present starch gel is the most popular support medium for enzyme electrophoresis in population genetics and biochemical systematics studies. This is due to some critical advantages of starch gel over other support media.

The main advantage of this support medium is that starch gel blocks can be easily sliced into several gel slices. As many as eight to ten slices can be obtained from a gel block 1 cm thick. This affords a researcher the ability to reveal the genotype of an individual for a much larger number of genic loci in one run than is possible with any other support medium. This characteristic of starch gel is especially valuable for large-scale isozyme and allozyme screenings. It allows rapid assessment of the genetic composition of a population, and the multilocus identity of individuals.

Being a natural biological product, starch does not contain any undesirable admixtures capable of inactivating enzymes or causing in vitro generation of nongenetic secondary isozymes, as is characteristic of polyacrylamide gel. This simplifies isozymal patterns displayed on zymograms and makes them more easily interpretable in genetic terms, especially for those enzymes that are represented by multiple isozymal forms. The degree of resolution attainable by electrophoresis on a starch gel is exceeded only by that attainable with a polyacrylamide gel. A starch gel works well with samples the size of a fruit fly. Some zymographic techniques (e.g., those based on the use of the starch–iodine chromogenic reaction) were developed specifically for the starch gel.

At the same time, the starch gel has some disadvantages; however, they are not critical and do not considerably diminish its value for large-scale isozyme and allozyme surveys. When compared with polyacrylamide and cellulose acetate, the following disadvantages of the starch gel are usually listed:

1. Different lots of commercially available hydrolyzed starch supplied by different (and even the same) manufacturers usually differ in composition and may contain differing proportions of amylose and amylopectin, which can affect gelling ability and resolution. Thus, it is essential that each new lot of starch be thoroughly tested in order to calibrate it before use.

2. Starch gel is not translucent and so not suitable for direct quantitative measurement by densitometry. However, it can be relatively easily rendered transparent by soaking it in hot (70°C) glycerol for a few minutes, or by soaking it first in water:glycerol:acetic acid (5:5:1 by volume) and then overnight in pure glycerol. The lack of uniformity of the starch gel matrix also makes it less suitable for densitometric quantitation than the polyacrylamide gel. Again, this disadvantage is not critical for population genetics surveys because the great majority of electrophoretically detectable intra- and interspecific allozymic and isozymal differences are qualitative rather than quantitative.

3. Conventional starch gel stains usually involve volumes of 25 to 50 ml, which is about one order more than is required for staining of cellulose acetate gels of the same size. The amount of staining solution, however, can be reduced considerably by the use of an appropriate procedure for stain application. For example, only 6 ml of the stain is just enough to cover the cut surface of the gel (30 × 20 cm) if applied dropwise with a Pasteur pipette.

4. Starch gel is more friable than polyacrylamide or cellulose acetate gels and is not easy to handle; however, there are usually no serious problems with handling starch gel for those who have spent a day practicing.

The choice of the most appropriate support medium is a very important step in any survey that uses enzyme electrophoresis. Usually no single support medium is a priori superior to any other. Each of the three media discussed above has its own particular characteristics that should be taken into account, depending on the problem to be solved, the biological object to be used as the enzyme source, the battery of enzymes to be analyzed, the resources available to the researcher, and other factors and circumstances. The correct choice of support medium will allow one to save time and money and to obtain more adequate data for solving the problem under investigation.

**Strategies of Gel Staining**

Several important questions that should be optimally resolved usually arise in connection with staining electrophoretic gels. The main ones are:

1. The choice of a more appropriate zymogram method, when more than one detection method is available for the enzyme under question
2. The choice of procedure for the faster and more correct preparation of functional staining solution
3. The choice of the mode of application of staining solution to the electrophoretic gel
4. The choice of methods allowing an increase in the staining intensity of the enzyme activity bands
5. The choice of the adequate staining of control gels for the assurance that the chosen zymogram method is specific for the enzyme under analysis

Unfortunately, each choice depends on many factors that may not be predicted with certainty beforehand. The most important characteristics of these factors are given below, with the purpose of allowing one to choose an optimal strategy of gel staining depending on the circumstances.
The Choice of Zymogram Method

This book comprises more than 900 different zymogram methods developed for more than 400 different enzymes. Thus, for many enzymes two or more principally different zymogram methods are available. Different methods developed for the same enzyme may differ in:

- Their applicability to a certain support medium
- Sets of reagents and special equipment needed
- Sensitivity
- Stain compatibility in double staining of the same gel
- Time and labor demands
- Methods of quantification of the resulting zymograms
- Sharpness and stability of stained bands
- Modes of band registration and zymogram preservation
- The cost of information obtained, etc.

These differences should be taken into account when making the choice of the more optimal zymogram method, depending on species and tissues that are thought to be used as the enzyme source, the support medium that is planned to be used or has already been chosen, the problem under study, the resources available, the kind and quality of information that must be obtained, etc. It is obvious that in each situation the choice of an optimal zymogram method will be the result of a complex compromise.

Preparation of Staining Solution

Each staining solution is buffered with the staining buffer at a specific pH. The pH value of the staining buffer used for the enzyme stain is a compromise between (1) the pH optimum of the enzyme activity, (2) the pH optimum of the staining reaction(s) used in the detection method, (3) the pH value of the buffer used for preparation of the gel, and (4) the pH optimum of the linking enzymes, if those are involved in staining reaction(s).

Many substrates (e.g., those used by dehydrogenases) are acids. In such cases stronger buffering capacity is required. Therefore, the use of high-concentration staining buffers is recommended. In some cases the substrates are such strong acids that their solutions must be prepared and brought to neutral pH before being added to staining solutions. Some commercially available substrates are unstable or very expensive. Solutions of such substrates can be prepared enzymatically in the laboratory. An example is gyceraldehyde-3-phosphate (see 1.2.1.12 — GA-3-P, Method 1). It is a good practice to add substrates and other ingredients to staining buffers, but not vice versa. This prevents the possible sharp decrease of the pH value of the staining mixture to a level at which the enzyme analysis will not function or other ingredients of the stain will be inactivated. For example, the reduced forms of NAD and NAD(P) cofactors denature in acid conditions very quickly. Dry chemicals should be taken out of the refrigerator or freezer about half an hour before preparation of staining solutions. This lets them warm up and prevents condensation. It is a general rule to add ingredients to the staining buffer in the sequence given in the recipe of the stain. The intermediate and final pH values should be checked for staining solutions that include acids or that are prepared for the first time.

For large-scale population surveys of isozymes, the preparation of stock solutions of reagents that are used regularly is recommended. The use of stock solutions allows one to prepare staining solutions quickly and accurately, to use only minimal quantities of expensive reagents, and to reduce the number of times the refrigerated chemicals are opened. This mode of staining preparation is especially valuable when large numbers of samples are being run for only a few enzymes. On the contrary, when as large as possible a number of enzymes is needed to be stained only once, the use of dry reagents is preferable. Many workers with much experience in stain preparation use an “analytical spatula” and simple eye control to weigh most dry reagents, unless they are very expensive or proportions of the stain ingredients are critical. For example, the amounts of NADH or NADPH added to the negative fluorescent stains are critical. If too little amounts are added, the background will not fluoresce sufficiently, but when too much is added, the detected enzyme (or NAD(P)H-dependent linking enzyme) will not be able to convert sufficient quantities of NAD(P)H into NAD(P) for nonfluorescent bands to become visible. Substrates at a high concentration can sometimes inhibit enzymatic activity. An example is the brain isozyme of octopine dehydrogenase in cuttlefish. When a negative fluorescent method is used to detect this enzyme, the use of a pyruvate concentration higher than 2 mM is not recommended (see 1.5.1.11 — ONDH, Method 2). Again, special care should be taken with some couplers and dyes that can display inhibitory effects on catalytic activity of some enzymes when they are above certain critical concentrations or even at relatively low levels. Examples are some diazo dyes, which have inhibitory effects on acid phosphatase (see 3.1.3.2 — ACP, Method 3, Notes), and the PMS–MTT system, which inhibits some dehydrogenases (e.g., see 1.1.1.22 — UGDH; 1.1.1.138 — MD(NADP)). Many of the staining mixtures tolerate, to some extent, variations in the amounts of their ingredients. It should be remembered, however, that use of substrate concentrations that are only somewhat lower than saturation levels can cause weak staining of enzymatic bands. On the other hand, substrate concentrations that are sufficiently higher than saturation levels usually do not reduce the staining intensity of enzymatic bands. Therefore, when unique and precious material is analyzed or when the optimal substrate concentration for the enzyme under analysis is not known, the use of higher substrate concentrations is recommended. The same is true for linking enzymes. The concentration of linking enzymes should also be increased when their activity is decreased as a result of storage. However, when a particular staining solution is used regularly, the possibility of reduction of the quantity of expensive ingredients usually exists. The recipes of staining solutions presented in this part often involve the addition of excess amounts of...
reagents. Thus, it is usually possible to reduce the amounts of the more expensive reagents, and thus the total costs of stains.

As a rule, the use of allozymes and isozymes as genic markers does not require quantitative measurements. Thus, the exact quantitative standardization is usually not necessary when preparing the same staining solution to detect allele frequencies or to compare isozyme patterns in different populations or in samples taken from the same population at different times.

It is a general rule that staining solutions should be prepared as quickly as possible and just before use. The speed of the stain preparation is often more important than the precision. This is especially true for large-scale surveys in which many enzymes are to be stained. The speed of stain preparation may in some cases be the most important limiting condition, essential for the production of functional staining solutions.

The main limitation of the use of reagents as stock solutions is their instability. Stock solutions usually involve only those reagents that are stable in solutions for at least several weeks. When two or more reagents have been mixed in a solution, the stain should be further prepared as quickly as possible. On the other hand, the mixtures of dry reagents essential for visualization of some enzymes can be stored in a refrigerator for a long time while being desiccated. The use of dry reagent mixtures is advantageous for electrophoretic enzyme assays that are to be carried out under field conditions. At present some producers are beginning to supply enzyme detection kits that are ready for use. For example, Innovative Chemistry, Inc. (P.O. Box 90, Marshfield, MA 02050) supplies kits suitable for detection of 18 different human enzymes. The only thing that must be done with the kit is to reconstitute one vial of enzyme reagent with a certain volume of deionized water and to pour the resulting staining solution over the surface of the electrophoretic gel.

It should always be remembered that the use of reagents (especially substrates and linking enzyme preparations) of high purity will allow one to avoid many problems caused by the use of low-quality reagents containing concomitants, which can interfere with other compounds and make the staining mixture nonfunctional or result in development of artifactual or nonspecifically stained bands. In many cases the cost of high-quality reagents is of secondary concern, because the most expensive “ingredients” in enzyme electrophoretic surveys are labor, time, and the precious collected material.

Amounts of staining solutions sufficient for gel staining vary considerably, depending on the size of the gel to be stained and the mode of application of the staining solution to the gel.

**Modes of Application of Staining Solutions**

Staining solutions can be applied directly to the surface of electrophoretic gels or used as filter paper or agar overlays. A standard staining solution method is the easiest and the most widely used. It consists of placing an electrophoretic gel (or gel slice) in a special staining tray, adding the staining solution until the gel is completely covered by fluid, and incubating the gel at room temperature or 37°C (usually in the dark) until enzyme activity bands are visible. Specifically designed staining trays made of glass or Plexiglas are usually used for this purpose. It is better to use a staining tray that exactly fits the size of the gel to be stained. If the trays for gel staining are larger than the gel, the amount of staining solution will have to be increased. This is not desirable because stains are usually expensive. For the staining tray that exactly fits the size of the 300 cm² gel, only about 50 ml of staining solution is needed to cover the top surface of the gel with fluid. However, this method is not always practical since many stains include reagents that are too expensive to be maintained at effective concentrations in large volumes. The problem can be overcome by preparing the concentrated staining solution in a small total volume that is just enough to flood the gel surface if applied dropwise with a pipette. Automatic adjustable-volume pipettes with disposable plastic tips or simple Pasteur pipettes are usually used for this purpose. The stain diffuses directly into the gel where the visualization reaction takes place.

When the dropwise application method is used, only about 3 ml of staining solution is needed to stain a gel of 300 cm² in size. Another method of application may also be recommended. It comprises placing a glass rod on the edge of the gel, pouring the staining solution on the gel above the glass rod, and spreading the solution evenly over the gel surface with the glass rod. When dealing with cellulose acetate gels, small amounts of staining solution can be applied by spreading it evenly on the glass plate to the width of the gel, and subsequent spreading of the stain over the gel surface by placing the gel, with the porous side down, onto the solution, avoiding the formation of air bubbles between the gel and glass plate. Methods that use small amounts of concentrated staining solutions are applicable to cellulose acetate gels and cut surfaces of starch and (less frequently) polyacrylamide gels. It is important to point out that application and spreading of small amounts of staining solutions should be done as quickly as possible to prevent uneven entrapment of the fluid by the gel matrix and subsequent uneven staining intensity of the enzyme activity bands in different parts of the gel. After the stain is absorbed (usually after 1 to 5 min) the gel should be covered with a sheet of plastic wrap sufficiently large to fully enclose the gel and protect it from drying during incubation.

Many stains, predominantly those that are based on positive or negative fluorescence, are applied to the gel using the filter paper overlay method. This method requires considerably less stain than the standard staining solution method based on the use of staining trays and requiring the immersion of the whole gel into the staining solution. When the time period of gel incubation is expected to be longer than 30 min, the paper–gel sandwich should be covered with a sheet of plastic wrap of appropriate size. A filter and chromatographic paper (Whatman No. 1 and 3MM) are usually used in this method. Cellulose acetate strips are also used sometimes and work even better than filter paper, but they are more expensive. The filter paper overlay method is useful for negative staining of enzymatic bands using negative fluorescent stains containing NAD(P)H coupled with subsequent counterstaining of the gel with a PMS–MTT mixture. In this case the areas of the gel occupied by the enzyme are indicated by achromatic bands on a blue background of the gel and paper. Moreover, the precipitation of blue formazan in the
paper is preferential. Thus, the stained paper may be easily washed, fixed, dried, and stored for a permanent record.

Staining solutions can also be applied as a 1:1 mixture with 2% molten agar (or agarose) cooled to 50 to 60°C. After preparation the mixture is quickly poured uniformly over the gel and allowed to cool and solidify. It should be remembered when using this method that if the agar solution is too hot, it can inactivate some reagents, e.g., linking enzymes, but if it is too cold, it can solidify just in the process of mixing with the staining solution. The agar overlay method has the advantage of bringing the reagents into intimate contact with the enzyme molecules to be stained for, and it also prevents diffusion of the stained bands, especially when the final product of the enzyme-visualizing reaction is soluble or when coupled enzymatic reactions generating soluble intermediates are involved in the enzyme-visualizing mechanism. Usually, the bands of enzymatic activity are well visible on the transparent agar layer. It can easily be taken off from the electrophoretic gel and the developed bands quantified by a scanning densitometer. Placed on a sheet of filter paper and dried, the developed agar layer may be stored for a long time and used as a permanent record of the zymogram. The agar overlay method is the only method suitable for detection of enzymes on nontransparent gels by two-dimensional gel spectroscopy. At present this method is one of the most popular methods of stain application in starch gel electrophoresis of enzymes.

The reactive agarose plate method is a modification of the agar overlay method. In this method 1% agarose solution containing all reagents needed for visualization of the enzyme activity is poured into a tray that fits the size of the electrophoretic gel to be stained. After agarose solidification the electrophoretic gel is laid over the agarose in order to expose the reactive agarose to the gel-entrapped enzyme. This method is used for visualization of enzymes by bioautography. Thin, substrate-containing agarose plates are also widely used for detection of many hydrolases with depolymerizing activity.

Each of the three main methods of application of staining solutions described above has its own advantages and disadvantages that depend on the type of support medium used for enzyme electrophoresis, the zymogram method chosen for visualization of the enzyme activity bands, the character and quality of information desired, and financial resources available. In general, however, the agar overlay technique gives better results than any other method and is always preferable when isozymes with high and low activity are presented on the same gel.

Modes of Enhancement of Staining Intensity of Enzyme Activity Bands

These modes can be divided into two main groups. The first group is represented by methods that are applicable before the procedure of detection of enzyme activity bands. It includes methods of protection and activation of enzymes during preparation of enzyme-containing samples and their electrophoretic run. Some modifications of the sample application procedure can increase the sample amount applied onto the gel, and thus increase the enzyme amount in the gel and the staining intensity of enzymatic bands on zymograms. These methods, however, have no direct relation to the procedure and mechanisms of the enzyme visualization and so are beyond the scope of this book. If interested, the reader may find more detailed information on the matter in other manuals on enzyme electrophoresis.\(^1,2,4-6,9\)

The second group comprises methods that are applied during or after the procedure of visualization of enzyme activity bands. One of these methods is the so-called postcoupling technique. It is recommended for use when the staining solution contains a particular reagent(s) that inhibits catalytic activity of the enzyme to be stained. For example, acid phosphatase is inhibited by some Fast diazo dyes (see 3.1.3.2 — ACP, Method 3, Notes), while some dehydrogenases (see 1.1.1.22 — UGDH; 1.1.1.138 — MD(NADP)) are supposed to be inhibited by PMS or MTT, or both. In such cases all reagents except the potential inhibitor should be applied to the gel at the first step in the usual way. After incubation for an appropriate period of time (depending on the expected activity of the detected enzyme), the remaining reagent should be added to the stain. The main disadvantage of the postcoupling technique is that it is not always possible to monitor the intensity of the visualizing reaction over time. Indeed, the product of the phosphatase reaction, naphthol, becomes visible only after coupling with diazo dye. Fortunately, the reaction catalyzed by NAD(P)-dependent dehydrogenases can be monitored under a UV lamp because the reaction products NADH or NADPH fluoresce in long-wave UV light. Thus, when the bands of NAD(P)H fluorescence are well developed, a PMS–MTT mixture should be added to the stain to make dehydrogenase activity bands visible in daylight. Another disadvantage of the postcoupling technique is the diffuse character of the developed enzymatic bands. This is because of relatively small sizes of enzyme product molecules, which readily diffuse through the gel matrix before insoluble colored precipitates are formed.

Some NAD(P)-dependent dehydrogenases are activated by manganese ions. When the PMS–MTT system is used to detect such enzymes, weak staining intensity of the enzymatic bands can be observed because of the inhibitory effect of manganese ions toward PMS as an electron acceptor (e.g., see 1.1.1.42 — IDH, Method 1, Notes). To overcome this problem, diaphorase should be used in place of PMS (see 2.4.1.90 — AGS, Method 1, Notes), or the amount of PMS included in the stain should be increased several times. Another way is to observe the enzyme activity bands in long-wave UV light after incubation of the gel in staining solution lacking PMS and MTT. When applying PMS–tetrazolium stains it should also be kept in mind that PMS is not stable at high-alkaline pH values.\(^10\) This can cause weak staining intensity of activity bands of dehydrogenases with high pH optima or when staining solutions with erroneously high pH values are used. The positive fluorescent methods may be preferable over PMS–tetrazolium methods when the endogenous enzyme superoxide dismutase (also known as tetrazolium oxidase) comigrates with NAD(P)-dependent dehydrogenases and considerably reduces or even fully prevents development of their bands via the PMS–tetrazolium stain.\(^11\)
It may sometimes be desirable to increase the concentration of certain reagents (usually substrates, cofactors, and linking enzymes) in order to enhance the intensity of staining of the enzyme activity bands. The use of the agar overlay method for detection of weak-activity enzymes on cellulose acetate gels is preferable because it allows not only an increase in the quantities of reagents applied, but also a prolonging of the gel incubation time.

In some cases weak staining of enzyme activity bands may be the consequence of inhibitory action of the enzyme reaction product toward the detected enzyme. To increase the speed of the enzyme reaction the product of enzymatic reaction should be trapped. An auxiliary enzyme that utilizes the product of the detected enzyme as its own substrate can be used for this purpose (see 2.6.1.1 — GOT, Method 4, Notes). Some original zymographic methods are based on the same mechanism (e.g., see 5.4.2.4 — BPGM). In some cases the excess products may be trapped and the intensity of band staining enhanced by the use of specific reagents. For example, such carbonyl-trapping reagents as hydrazine hydrate and aminooxycetic acid are used to trap 2-oxoglutarate with the purpose of displacing the glutamate dehydrogenase reaction toward the production of NAD(P)H, which serves as a fluorescent indicator of the enzyme reaction.\(^2\) As a result, the accelerated production of NAD(P)H intensifies the staining of glutamate dehydrogenase bands by the tetrazolium method (see 1.4.1.2 – 4 — GDH, Method 1, Notes).

The apparent activity of some enzymes can be doubled by linking it to two sequential reactions or to dehydrogenase/isomerase/dehydrogenase 2 sequential reactions, which produce two molecules of a reduced or oxidized dehydrogenase cofactor. For example, two NADP-dependent sequentially acting dehydrogenases — glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase — are used to double the production of fluorescent NADPH in the positive fluorescent method developed for detection of galactokinase (see 2.7.1.6 — GALK, Method 3). Another example is the glycerol-3-phosphate dehydrogenase/triose-phosphate isomerase/glyceraldehyde-3-phosphate dehydrogenase system of sequentially acting auxiliary enzymes, which can work in both directions and may be used to double the production of NADH (the forward direction) or NAD (the backward direction). Examples of the use of this linking enzyme system are detection methods for glycerol kinase (2.7.1.30 — GLYCK, Method 2), phosphoglycerate mutase (5.4.2.1 — PGLM, Method 1), and phosphoglycerate kinase (2.7.2.3 — PGK).

Enzymatic cycling is a method widely used for amplifying the signal of enzymatic reaction. This method uses two different enzymes coupled in the opposite direction, so that the substrate of one of them is the product of the other, and vice versa. Under such conditions, the reaction turns out with no consumption of recycling substrates, while other products of enzymatic reactions are accumulated with each turn of the cycle. The use of enzymatic cycling is of special value for detecting enzymes present in low concentrations and producing low levels of detectable signals. The method of enzymatic cycling was proposed for determining L-glutamate, which involves auxiliary enzymes L-glutamate oxidase (EC 1.4.3.11), alanine transaminase (EC 2.6.1.2), and horseradish peroxidase (EC 1.11.1.7).\(^3\) This method may be applied to detect enzymes producing L-glutamate (e.g., see 3.5.1.2 — GLUT, Other Methods, C; 4.1.3.27 — AS, Other Methods, B). ATP-ADP cycling reactions catalyzed by auxiliary enzymes adenylate kinase (2.7.4.3 — AK), pyruvate kinase (2.7.1.40 — PK), and hexokinase (2.7.1.1 — HK) are used coupled with auxiliary glucose-6-phosphate dehydrogenase (1.1.1.40 — G-6-PD) to detect 5'-AMP produced by 3',5'-cyclic-nucleotide phosphodiesterase (e.g., see 3.1.4.17 — CNPE, Method 3; 4.6.1.1 — AC).\(^4\) These reactions may be used to detect activity bands of other enzymes producing 5'-AMP, particularly amino acid–tRNA ligases (6.1.1.2 — TRL; 6.1.1.4 — LRL; 6.1.1.9 — VRL; 6.1.1.16 — CRL), using the second step of an aminocyl–tRNA ligase reaction.

Fluorescence of 4-methylumbellifereone, which is the product of hydrolytic cleavage of 4-methylumbellifereone derivatives by numerous hydrolases, may be enhanced considerably by treating the developed acidic gels with an alkaline buffer (pH 9 to 10) or ammonia vapor.

The miniaturization of developed polyacrylamide gels by treatment with hot (70°C) 50% (w/v) polyethylene glycol (PEG 2000) increases the sensitivity of enzyme detection methods five to ten times.\(^5\) Methanol-containing fixatives may be used to miniaturize (although to a lesser extent) developed starch gels (for details, see “Recording and Preservation of Zymograms,” below).

Specificity of Zymogram Methods and Some Related Problems

Most of the zymogram methods presented in this part are enzyme specific. However, some methods can detect more than one enzyme. This results from the ability of some enzymes to utilize one or more of the applied reagents, or to use certain buffer constituents coupled with the applied staining solution reagents. Another reason for the development of unexpected enzymatic bands is the combined effect of two comigrating endogenous enzymes, one of which acts as a linking enzyme for the other in the presence of certain necessary reagents contained in the applied staining solution. Some enzymes can form complexes with cofactors or substrates that are stable during electrophoresis, or can use residual amounts of their substrates that are contained in some reagents as concomitants. Therefore, when other reagents needed for visualization reactions of such enzymes are available from applied staining solutions, the nonspecifically stained additional bands will appear. Finally, several cases are known where nonenzymatic protein bands or even nonprotein bands are developed by the use of certain stains.

Thus, when a new enzyme detection method is developed or when an approved method is used to detect a certain enzyme from a new enzyme source, it should be tested for its specificity for the enzyme under analysis. This is especially necessary for complex methods involving one or more reactions catalyzed by exogenous linking enzymes. A standard procedure for testing specificity of the method to be used includes a series of experimental
stainings in which each individual constituent of the stain is omitted one at a time in order to determine if any stained bands appear after treating control gels with incomplete stains. The specificity of the enzyme detection method can also be tested by the use of alternative methods, if they are available for the enzyme under investigation, or if they may be devised by using a backward reaction, or by using a principally different mechanism of visualization, which involves different substrates, cofactors, sets of linking enzymes, etc.

Non-specific staining of additional unexpected bands on zymograms of some enzymes is illustrated by the following examples.

Bands of adenylate kinase (see 2.7.4.3 — AK, Method 1) can develop on zymograms of pyruvate kinase (2.7.1.40 — PK, Method 2), creatine kinase (2.7.3.2 — CK, Method 2), and arginine kinase (2.7.3.3 — ARGK, Method 1). This is because all the reagents needed for AK detection (i.e., ADP, glucose, NADPH, MTT, PMS, hexokinase, and glucose-6-phosphate dehydrogenase) are contained in staining solutions used to detect the enzymes listed above.

It is well established that adenylate kinase molecules have a monomeric subunit structure that is highly conserved during organismal evolution. In this connection, the description of unusual allozymic polymorphisms of dimeric adenylate kinase revealed in some invertebrate and vertebrate animals is the consequence of misleading interpretation of allozymic variation of nonspecifically stained bands of dimeric glucose dehydrogenase (see 1.1.1.47 — GD, Method 1), which is widely distributed among different animal groups. To be developed, this dehydrogenase requires just those reagents (i.e., glucose, NADPH, MTT, and PMS) that are present in the adenylate kinase stain.

When negative fluorescent methods are used to detect opine dehydrogenases (e.g., 1.5.1.11 — ONDH, Method 2; 1.5.1.17 — ALPDH, Method 2), the bands caused by lactate dehydrogenase activity can also develop in some invertebrate species containing all these dehydrogenases.

Some phosphatases of invertebrates and vertebrates can use phosphoenolpyruvate as a substrate and thereby produce pyruvate. Thus, the bands of phosphoenolpyruvate phosphatase activity can develop on pyruvate kinase zymograms obtained by a negative fluorescent method (2.7.1.40 — PK, Method 1, Notes) and on zymograms of many other enzymes obtained using detection methods involving pyruvate kinase and lactate dehydrogenase as linking enzymes (e.g., see 2.7.3.2 — CK, Method 1; 2.7.3.3 — ARGK, Method 2; 2.7.4.3 — AK, Method 2; 2.7.4.4 — NPK; 2.7.4.8 — GUK, Method 1; 2.7.6.1 — RPPPK, Method 1).

It has been found that human aconitase isoforms can sometimes appear on zymograms obtained using staining solution lacking both the enzyme substrate, cis-aconitate, and the linking enzyme, isocitrate dehydrogenase. This unexpected phenomenon was shown to occur only when electrophoresis was carried out using a citrate-containing buffer. It is known that aconitase is capable of catalyzing two reactions:

1. citrate = cis-aconitate + H₂O
2. cis-aconitate + H₂O = isocitrate (see 4.2.1.3 — ACON)

Thus, aconitase can use citrate from a citrate-containing gel buffer as a substrate and thereby produce isocitrate. When aconitase comigrates with isocitrate dehydrogenase, staining occurs at those gel sites where two endogenous enzymes occur together. When one or both of these enzymes are polymorphic, the individual differences in development of unexpected additional bands may be observed, because under these conditions both enzymes migrate to overlapping positions only in some individuals.

When a standard staining solution method is used to detect hexokinase (see 2.7.1.1 — HK), bands of 6-phosphogluconate dehydrogenase (1.1.1.44 — PGD) may also develop, especially when the gel is subjected to prolonged incubation. This is because the linking enzyme glucose-6-phosphate dehydrogenase utilizes the glucose-6-phosphate generated by endogenous hexokinase and produces glucono-1,5-lactone 6-phosphate, which spontaneously turns into 6-phosphogluconate, the substrate of 6-phosphogluconate dehydrogenase. The molecules of this substrate freely diffuse in the liquid stain and become available for endogenous 6-phosphogluconate dehydrogenase, for which bands may further develop in the presence of NADP, PMS, and MTT involved in the hexokinase stain. Of course, this is the case only when an NADP-dependent glucose-6-phosphate dehydrogenase preparation is used in the hexokinase stain.

Additional unexpected bands are frequently observed on zymograms of NAD(P)-dependent dehydrogenases. These bands develop even when staining solutions lacking any dehydrogenase substrates are used. This effect has been referred to as “nothing dehydrogenase” (see 1.X.X.X — NDH). Two main reasons are known to cause the NDH phenomenon: (1) binding of endogenous substrates by some dehydrogenase molecules, and (2) contamination of some commercial preparations with substances that serve as substrates for some dehydrogenases. Two enzymes — alcohol dehydrogenase and lactate dehydrogenase — usually are the most probable candidates for NDH. The bands of NDH activity may be easily identified by their repetitive occurrence on gels stained for different dehydrogenases or other enzymes detected using linked dehydrogenase reactions.

Some nonenzymatic proteins can also cause the development of false bands on gels specifically stained for some enzymatic activities. At least four examples are known. The first one concerns SH-rich proteins, which are capable of reducing a tetrazolium salt even in the absence of NAD(P) and PMS. The second example is the interference of albumins with the starch–iodine color reaction, resulting in the development of false amylase bands on zymograms obtained by the negative starch–iodine method. Albumins are also known to be able to cause the appearance of additional artificial bands on acid phosphatase and alkaline phosphatase zymograms obtained with the azo coupling methods. In the last case it is shown that the alkaline phosphatase–like activity in the albumin zone is an artifact due to a bilirubin–albumin complex that nonspecifically couples with a diazo compound.
When the samples used for electrophoresis contain high levels of endogenous reduced glutathione or other sulphydryl compounds (e.g., 2-mercaptoethanol or dithiothreitol) added to the samples prior to electrophoresis to stabilize or activate the enzyme under analysis, the appearance of additional stained bands should be expected on zymograms obtained by the use of staining solutions containing MTT–PMS. The bands caused by electrically neutral 2-mercaptoethanol and dithiothreitol molecules are usually detected in the cathodal part of starch gels because of the effect of electroendosmosis. Negatively charged molecules of reduced glutathione usually migrate to the very anodal part of the gel. The bands caused by all these thiol reagents are monomorphic, diffuse, and stained fairly weakly, and are observed on zymograms of different enzymes detected for the same samples by tetrazolium methods.

Two different enzymes with overlapping substrate specificity are able to catalyze the same reaction and thus to display their activity bands on the same zymogram. For example, it was shown that some isozymes of alcohol dehydrogenase from Drosophila melanogaster also oxidize L(+)-lactate or D(–)-lactate with NAD as a cofactor, and intraspecific electrophoretic variation observed on lactate dehydrogenase zymograms could be attributed to the presence of alcohol dehydrogenase. This phenomenon was called pseudopolymorphism. Another example is the existence of some isozymes in the snail Cepaea nemoralis that can utilize either malate or lactate as a substrate, converting both into pyruvate. A special study has shown that there are many enzymes that are listed in the enzyme list under different code numbers, although they are coded by one and the same gene in a wide range of organisms that represent phylogenetically distinct groups. As to dehydrogenases, identical allozymic patterns were revealed on zymograms of octanol dehydrogenase (1.1.1.73 — ODH), formaldehyde dehydrogenase (1.2.1.1 — FDHG), and D-lactaldehyde dehydrogenase (1.1.1.78 — DLADH) from the sipunculid Phascolosoma japonicum. Identical allozymic variations were observed on zymograms of ODH and FDHG in each of four bivalve species of the genus Macoma that were examined. In the phoronid Phororopsis harmeri the allozymic pattern of the ODH-1 isozyme was found to be identical to those of DLADH, while the allozymic pattern of the ODH-2 isozyme was identical to that observed on the FDHG zymogram. The same allozymic patterns were revealed on ODH, FDHG (FDHG-2 isozyme), and DLADH zymograms in the mushroom Boletus edulis. All these examples are evidence that certain dehydrogenases that are believed to be different proteins are really a single enzyme protein with broad substrate specificity. The same bands can also be observed on zymograms of acid and alkaline phosphatases, and on zymograms of different peptidases. It was shown, for example, that numerous phosphatases from the sipunculid intestine display identical allozymic patterns in the same sample of conspecific individuals. Similar results were obtained with hexokinase (2.7.1.1 — HK) and fructokinase (2.7.1.4 — FK) from a mushroom, nemertine, domestic fly, starfish, and sea urchin. Thus, a number of cases are known where enzymes that are believed to be different are encoded by a single genic locus, and the possibility exists to erroneously score the same locus twice or even more.

**RECORDING AND PRESERVATION OF ZYMOGRAMS**

There are at least three different methods of recording the enzyme patterns developed on electrophoretic gels: (1) schematic recording of zymograms, (2) photography of zymograms, and (3) the tracing of the band position on paper overlays or cellulose acetate gels. After recording, zymograms can be stored in special fixative solutions or dried. Schematic recording of zymograms involves a two-dimensional representation of the banding patterns observed on developed gels. Although such representation suggests some degree of simplification and subjectivity, it can be excused during routine surveys of well-known enzyme polymorphisms. Another technique that allows the keeping of a permanent record of the results of each electrophoretic experiment is photography. The main advantage of photographs is their objectivity. They are relatively rapidly produced and easy to store. Stained cellulose acetate and starch gels are photographed with uniform lighting from above, while stained translucent agar overlays and polyacrylamide gels are photographed on a light box with lighting transmitted from below. Sometimes special conditions are required for photographing stained gels. For example, polyacrylamide gels stained using the method of calcium phosphate precipitation should be photographed by reflected light against a dark background. In order to produce permanent records of indicator agar plates developed by the method of bioautography, an indirect lighting system is used that is a large version of the lighting system employed for photography of immunodiffusion plates. For photographing fluorescent bands (or nonfluorescent bands on fluorescent background), reflected UV light is used in combination with a yellow filter (e.g., Wratte Gelatin Filter No. 2E, Eastman Kodak Company). The exposure for fluorescent gels is usually 20 to 30 sec. The use of an automatic-exposure camera considerably facilitates the photographing of such gels. The use of a yellow filter also enhances the contrast of blue bands and is routinely employed in photographing all gels stained with tetrazolium stains. Black-and-white films such as Kodak Panatomic-X, Plus-X, or Technical Pan are recommended for photographing bands visible in daylight. For photographing fluorescent bands (or nonfluorescent bands on fluorescent background), high-speed films such as Polaroid film type 55 or 57 or regular film ASA 400 are recommended. Color slides of good quality can be obtained with Kodachrome ASA 25.
over the bands with a ballpoint pen or a marker. This technique is especially useful for recording electrophoretic patterns obtained using fluorescent detection methods or for recording bands that fade after a short period of time.

Stained gels can be fixed and stored for a permanent record or dried after appropriate treatments. Cellulose acetate gels are usually fixed in 5% formalin or 10% acetic acid for 10 to 15 min and then stored in 10% glycerol or placed in airtight plastic bags for convenient handling. Starch gels are usually fixed in 7% acetic acid or 25% ethanol and then stored in special trays filled with a 5:5:1 (v/v) mixture of methanol:water:acetic acid. This fixative toughens and shrinks the gels and makes them opaque. The alcohol gel wash recommended for starch gels consists of a 5:4:2:1 (v/v) mixture of ethanol:water:acetic acid:glycerol.32 It not only stops the staining but also toughens the gel and helps bleach out some of the background. The use of 50 to 100% glycerol does not harden the starch gel as the alcohol-containing fixatives do; however, it helps to maintain gel integrity and makes the gel translucent and suitable for densitometric measurements. A 5% glycerol–7% acetic acid mixture is also frequently used for fixing and storing stained starch gels. Fixed gels can also be stored in airtight plastic bags. Stained polyacrylamide gels are usually fixed in 7% acetic acid or glycerol–acetic acid mixtures. The fixed gels can then be stored in a refrigerator for several months.

The method that gives a more permanent record of isozyme patterns is gel drying. For example, a simple method was developed for the preparation of fully transparent, flexible, dry sheets of stained starch gels.33 In this method the stained gel is kept in 7% acetic acid, washed twice with water to remove excess reagents, and kept in 5% glycerol for 15 to 30 min. The gel is then placed on a glass plate of appropriate size, previously covered with a second cellophane sheet. Both sheets must be sufficiently large to allow at least a 2-cm margin on all sides of the glass plate. Both cellophane sheets must be soaked in 5% glycerol solution for 15 to 30 min before use. The formation of air bubbles between the gel and the sheets should be avoided. The four edges of the double cellophane are then folded over the back of the glass plate, the excess amount of glycerol solution removed by filter paper, and the processed plate dried at room temperature or at 60 to 80°C. After the gel and cellophane have been dried, the cellophane sheets are cut with a razor blade, about 1 cm from the border of the gel. The dried transparent and flexible gel covered with cellophane can then easily be stripped off the glass plate. This method allows storage of stained starch gels for up to 9 years with full preservation of isozyme patterns. A similar procedure may be used to obtain dried polyacrylamide slab gels, except 65% methanol containing 0.5% glycerol should be substituted for the 5% glycerol solution. Dry polyacrylamide slab gels suitable for autoradiographic detection of [14C]- and [3H]-labeled zones can also be obtained by placing the thin slab onto a glass plate large enough to allow the formation of at least a 2-cm margin all around the gel, subsequent covering of the gel with 2% agarose solution, and drying of the “sandwich” slowly and evenly, at first using an infrared lamp and then at room temperature. The stained polyacrylamide slab gels pretreated for 1 h with a 7.5% acetic acid–1.5% glycerol solution also can be dried after being covered with a 5% aqueous solution of gelatin or sprinkled with aquecide I, II, or III.34 The instruction for a treatment that converts cellulose acetate gel into a film similar to cellophane is supplied with Cellogel by the manufacturer.

The above-described techniques and facilities developed to record and preserve zymograms are now being intensively substituted with digital photo cameras, scanners, and PC softwares to produce, process, and store images of zymograms.

**RESOURCE-SAVING STRATEGIES**

The cost of information obtained by enzyme detection methods can be a limiting factor in some electrophoretic surveys of enzymes, e.g., in large-scale population studies. Several different ways exist to overcome this problem.

As outlined above, the choice of adequate and less-expensive zymogram methods, the reduction of quantities of the most expensive reagents in the staining solutions used, and the use of the most economical modes of stain application can help one to decrease expenses considerably. For example, when detecting glutamate–oxaloacetate transaminase (see 2.6.1.1 — GOT) in a population survey there is no need to use quantitative enzymatic Method 4 because the routine diazo coupling Method 1 gives quite satisfactory results and is about two orders less expensive than the former one. For many histochemical detection methods the expenditure of staining solutions can be decreased more than ten times when applied on a filter paper overlay or dropwise. Some other specific tactics also can be used to save resources during electrophoretic studies of enzymes. The most important of them are (1) simultaneous detection of two or even more enzymes on the same gel, (2) successive detection of different enzymes on the same gel, (3) reusing of staining solutions, (4) the use of several origins on the same gel, (5) multiple replication of running gels by electroblotting, and (6) the use of semipreparative gels as the sources of linking enzymes.

**Simultaneous Detection of Several Enzymes on the Same Gel**

Theoretically, any two or more different enzymes can be simultaneously stained on the same gel when their detection methods are based on the same visualizing mechanism.3 In practice, however, the combined stains are applicable only to those enzymes displaying nonoverlapping isozymal spectra under the electrophoretic conditions used.

Good examples of stain compatibility based on identity of the detection mechanisms involved are many NAD(P)-dependent dehydrogenase stains involving the PMS–tetrazolium system and some other stains using any NAD(P)-dependent dehydrogenase as a linking enzyme to catalyze the last step in the visualizing reaction, i.e., the production of colored formazan.

Different dehydrogenase stains are also compatible when positive or negative fluorescent detection of their bands is based on an NAD(P)-into-NAD(P)H or NAD(P)H-into-NAD(P)
conversion mechanism, respectively, or when detection of some nondehydrogenase enzymes is based on this same mechanism implemented through a linked reaction catalyzed by an appropriate NAD(P)-dependent dehydrogenase.

A combination of stains for different hydrolases that use 4-methylumbelliferone derivatives as substrates is possible and can be successful, especially when hydrolases with similar pH optima are combined.

The main disadvantage of the stain combinations listed above and of some other possible combinations is that different sets of bands displayed by different enzymes are of the same color and hence may be confused. Thus, before combining, different enzymes should be stained on separate gels electrophoresed under the same conditions or stained on different gel slices obtained from the same gel block to identify relative positions of bands displayed by enzymes, the stainings of which are supposed to be combined. However, when compatible stains of polymorphic enzymes with different subunit structures of their molecules are combined, there is no need to stain enzymes separately because allozyme patterns displayed by different enzymes can be easily identified through the number of allozymes displayed in heterozygous individuals.2,3

The combination of compatible enzyme stains is particularly suited to large-scale population surveys where the range of expected genotypes is known. This tactic also allows staining of more enzymes when only extremely small sample volumes are available that are not sufficient for loading several different gels. It not only saves expensive reagents involved in the process of enzyme staining but also decreases the gel and time expense.

Simultaneous staining of different enzymes on the same gel is possible even when their detection methods are incompatible. This can be achieved through application of incompatible staining solutions in different strips of agar or acetate cellulose overlaid on different parts of the gel. The tactic of by-strip application is justifiable only when gel areas occupied by different enzymes to be detected were identified as a result of previous stainings of enzymes on separate gels. The use of the by-strip application method prevents mixing of incompatible stains, and interaction or interference of ingredients from different stains can occur only in a narrow contact zone where ingredients from different stains mix due to restricted diffusion.

Successive Detection of Different Enzymes on the Same Gel

This approach can be implemented not only when the stain compatibility of different enzymes is based on identity of mechanisms of their detection (i.e., in situations described above), but also when compatibility is based on tolerance of different detection mechanisms.4 The latter situation assumes that none of the reagents from the first stain interact or interfere with any reagent from the next one. In this case the activity bands caused by different enzymes usually are of different colors and so can be readily distinguished. A particular sequence of applied stains may not be reversible. For example, successful double stains are a purine-nucleoside phosphorylase tetrazolium stain (see 2.4.2.1 — PNP) followed by a glutamate-oxaloacetate transaminase stain based on the diazo coupling mechanism (see 2.6.1.1 — GOT, Method 1), and an esterase fluorescent stain (see 3.1.1 .. — EST, Method 2) followed by a peptidase stain involving L-amino acid oxidase and peroxidase as linking enzymes (see 3.4.11 or 13 .. — PEP, Method 1). In both cases the reverse sequence of stain application will not be successful simply because the products of the first-step staining reactions will mask the products of the second-step staining reactions.

In some cases different stains are incompatible because an ingredient of one stain is able to react with an ingredient in another. Examples are a glycerol-3-phosphate dehydrogenase tetrazolium stain (see 1.1.1.8 — G-3-PD) followed by a glycerol kinase tetrazolium stain (see 2.7.1.30 — GLYCK, Method 2), or a glucose–phosphate isomerase stain (see 5.3.1.9 — G-3-PD) followed by a mannose–phosphate isomerase stain (see 5.3.1.8 — MPI), where the substrates of the first stains will react with linking enzymes of the following ones. The same is true for negative fluorescent stains involving NAD(P)H followed by a tetrazolium stain where NAD(P)H of the first stain will interact with the PMS–tetrazolium system of the following one, and thus will result in formation of colored formazan across the whole gel surface. The problem of stain incompatibility can be overcome by careful washing of the gel after the first staining, with the purpose of removing soluble ingredients capable of interaction with ingredients in the following stain.5 This tactic, however, is applicable only when undesirable compounds of the first stain are soluble. Most suitable for implementation of this tactic are coarse-pored cellulose acetate gels, thin slices of starch gel blocks, and thin blocks of low-percentage polyacrylamide gels. The use of just those gels allows small molecules of undesirable reagents to diffuse readily into the washing solution, while large enzyme molecules remain in their final run position, being incorporated into the gel matrix. In some cases this tactic may be the only possible one for detecting different enzymes on the same gel when incompatible detection mechanisms are involved in different stains. An example is a situation when banding patterns produced by different incompatible stains overlap, and thus the by-strip application method cannot be used.

The successive detection of different enzymes using compatible stains may also sometimes be preferable because it does not require preliminary identification staining of different enzymes on separate gels. An example is a situation when the final detected products of successive stains are the same color and the banding patterns displayed by the different enzymes cannot be identified on the basis of differences of their subunit structures (e.g., when all enzymes are monomorphic or when their molecules are of the same subunit structure).

When compatibility of successive stains is based on the identity of the detection mechanisms involved, both reagent and gel saving may be achieved because only the addition of a specific substrate is needed for detection of each following enzyme. Only gel saving is achieved through double staining of the same gel if different detection mechanisms are involved in different successive stains. Again, successive double stainings are of great value when extremely small volumes of samples that
are not sufficient for loading more than one gel are available, and gel areas occupied by different enzymes overlap.

The Reuse of Staining Solutions

When a large-scale population survey is carried out using the standard staining solution method of enzyme detection, some staining solutions may be used repeatedly immediately after the first use, after 1 day of storage in a refrigerator, or after several days of storage in a freezer. The staining solutions that easily endure this tactic are those used for tetrazolium detection of numerous NAD(P)-dependent dehydrogenases and many other enzymes that are detected through the use of dehydrogenases as linking enzymes to produce a final visible product, formazan. The repeated addition of MTT or NBT and a linking enzyme (if involved) to the previously used staining solution is sometimes desirable. The tactic of reusing tetrazolium-containing stains is of great value, especially when expensive substrates and cofactors are involved in visualizing reactions. The potential reagent savings make this tactic a desirable addition to the resource-saving strategies of a laboratory.

The Use of Several Origins on One Gel

Two or more different sets of samples may be loaded in different origin positions on the same gel. This is a very useful tactic, especially when large-scale population screening of single-locus enzyme polymorphisms is carried out. The use of multiple origins is most effective for polymorphic enzymes with a small number of allelic variants of low electrophoretic mobility. Preliminary test runs may be needed to find optimal relative positions of origins to be sure that allozyme sets from different origins do not overlap. It should be taken into consideration that separation of the same allozymes started from different origins may be different. This disadvantage is especially pronounced when discontinuous buffer systems are used for electrophoresis. However, there are usually no problems with genetic interpretation of enzyme polymorphisms with known genotypes. The multiple-origin tactic is the most effective one in a search for rare enzyme polymorphisms with known genotypes. The multiple-origin tactic is the most effective one in a search for rare enzyme polymorphisms with known genotypes. The advantages of this tactic are the obvious cost savings in gel media, fine biochemicals, and time.

Multiple Replication of Electrophoretic Gels by Electroblotting

Electrophoretic transfer of enzymes from a single running gel onto immobilizing matrices (e.g., Zeta Pore or Hybond-N membranes and Whatman DE 81 paper) allows one to obtain multiple replicas, which can then be developed separately using different enzyme stains. It is important to remember when using this tactic that the faster-migrating enzymes should be detected on the first replica, while the slower-migrating ones should be on the last replica. Thus, use of the replication tactic requires preliminary knowledge of relative positions of isozymal sets displayed by different enzymes under analysis. Multiple replication and multiple successive staining tactics may be combined, resulting in a considerable increase in the number of different enzymes analyzed through one gel electrophoretic run. The electrophoretic transfer of enzymes requires additional expensive materials, special designs, and time. Thus, it is not as practical as some other resource-saving tactics described above. At the same time, it may be potentially very valuable when only extremely small volumes of each sample are available and obtaining additional material is very labor- and time-consuming, or impossible.

The Use of Semipreparative Gels for Production of Preparations of Linking Enzymes

A great number of enzyme-visualizing methods listed below in this part involve exogenous linking enzymes, some of which are not yet commercially available. Again, in some cases the purchase of even one vial of very expensive enzyme preparation may not be reasonable, e.g., when only one or a few gel stainings are supposed to be carried out. In such situations the semipreparative gel electrophoresis presents a good opportunity to obtain the enzyme needed in small but sufficient amounts and purity. In principle, all the enzymes that can be detected through any visualizing methods and thus located on electrophoretic gels may be used as linking enzymes. One critical condition must be fulfilled therefor: the preparation of the enzyme to be used as the linking one must be free of the enzyme to be detected. The use of the linking enzyme preparation contaminated with the enzyme under detection will result in background staining without any distinctly visible bands. The tactic of using semipreparative gels for the production of linking enzyme preparations was tested by the author and proved to be a good addition to hiszymographic "cookery."

The starch gel blocks routinely used for analytic electrophoresis may be easily adapted for semipreparative enzyme electrophoresis. After completion of electrophoretic separation the position of a desired enzyme on the gel is located by an appropriate detection method, and the gel area occupied by the enzyme is cut out. The use of starch gel as the supporting medium is preferable because a simple "freezing–squeezing" procedure may be used to remove the enzyme from the gel matrix. This procedure, for example, was successfully used by the author to obtain preparation of isocitrate dehydrogenase involved in a linking enzyme in an aconitase stain (see 4.2.1.3 — ACON, Method 1). A more effective method, electrophoretic elution, may also be used to remove enzymes from a gel matrix, but it is more time-consuming and requires an additional device.

An electrophoretic procedure very similar to that used for electrophoretic transfer of proteins may sometimes be even more effective than that described above. In this case proteins move in the transverse direction after being applied on the upper surface of a starch gel block by the agar overlay method. After an appropriate period of time electrophoresis is stopped. The position of the desired linking enzyme within the gel block is located by a specific detection method. For this purpose the gel slice obtained by transverse slicing through the gel block is used. A longitudinal slice is then made through the zone occupied by the enzyme, and two reactive gel plates with enzyme-containing cut
surfaces are obtained. The staining solution containing all necessary ingredients except the linking enzyme is applied dropwise to the surface of the gel to be stained. The reactive gel plates are then placed above, the cut surfaces down, avoiding formation of air bubbles. The linking enzyme molecules embedded in the gel matrix of the reactive plates usually work sufficiently well due to the diffusion of small molecules of reagents involved in the stain and those produced by the enzyme under detection. This variant of the method, for example, was successfully used by the author to obtain the reactive starch gel plates containing glucose–phosphate isomerase involved in the mannose–phosphate isomerase stain (see 5.3.1.8 — MPI) as the linking enzyme.

Of course, the use of semipreparative gel electrophoresis for production of auxiliary enzymes is not appropriate for large-scale isozyme survey; however, it provides a good opportunity for new developments in electrophoretic zymography. This tactic should not be considered now as an effective resource-saving one. Rather, it can be used to help develop and test new enzyme detection methods and thus is of great potential value.

**Troubleshooting**

During practical application of enzyme detection methods some problems concerning the enzyme staining originate from time to time. One may hope that a good knowledge of the detection mechanisms given in Part II will help to solve these problems. Nevertheless, some useful advice concerning stain troubleshooting is given below. These pieces of advice are organized into a list including the most common causes of stain failures and recommendations for their avoidance:

- **Some reagents are erroneously omitted from the staining solution:** Check the recipe. Add missing reagents or prepare a new solution.
- **Unstable reagents deteriorated during storage under inappropriate conditions:** First of all, check the reactivity of the linking enzymes, if involved, by a rough qualitative enzyme assay using appropriate staining solutions. Replace linking enzyme preparations if they have deteriorated. If the stain still does not work, check which reagents of the stain other than the linking enzymes are not functional. This may be done through simple analysis of functioning of other stains that include reagents common to the stain under question, or through one-by-one replacement of suspected reagents with fresh ones. If reserve vials of suspect reagents are not available, try to use suitable enzyme preparations and substrates to produce suspect reagents enzymatically. If available, use any alternative detection method to be sure that the enzyme under detection is active in your samples. Use control samples that show activity of the enzyme under detection with certainty. Follow storage instructions depicted on containers. Store most chemicals in a refrigerator or freezer in a special airtight box with a desiccant. Do not leave refrigerated chemicals open at room temperature. Hydrolysis following water condensation is particularly damaging to many compounds. To prevent condensation, take the chemicals out of the refrigerator about half an hour before use or warm the containers in your hands before opening. On the contrary, keep linking enzyme preparations at room temperature for as short a time as possible. Remember that some reagents are light sensitive and that high temperature also contributes to the breakdown of many reagents.

- **Intermediate or final pH value of staining solution deviates from that recommended in the recipe:** Check intermediate pH values during preparation of the staining solution and final pH before application. Prepare a new staining buffer if it has deteriorated. Use sodium or other salts rather than free strong acids or bases if they are involved in the stain. Always add reagents in the sequence given in the staining solution recipe.
- **Enzyme inhibitors are present in used water:** If the stain works well in laboratory conditions and does not work under field conditions, the bad quality of the water used is the first candidate for the reason of the stain failure. Use filtered rain or thawed snow water in the field and bidistilled or deionized water in the laboratory.

It also should be kept in mind that many errors that have been made during former stages of enzyme electrophoretic analysis (e.g., storage, preparation, and electrophoretic run of samples) manifest themselves only during the stage of enzyme detection. For example, the enzyme to be detected may be inactivated during tissue storage under inappropriate conditions (e.g., after freezing–thawing events) or may run off the anodal or cathodal end of the gel (e.g., when the enzyme moves quickly toward the anode or cathode or the running time is too long).

Some other hints for troubleshooting, especially for stages other than enzyme staining, may be found in the excellent handbook for allozyme electrophoresis by Richardson et al.4

**Safety Regulations**

A large array of compounds is used in enzyme-visualizing procedures. A number of these compounds are known to be carcinogens, skin irritants, or poisons. Many of these compounds have not even been tested yet for their long-term effects on human health and heredity. Thus, it is good practice for those dealing with such a diverse group of chemicals to follow the general safety codex given below:

- Treat all chemicals as potentially hazardous to your health.
- Wear gloves and a dust mask when handling chemicals.
- Do not breathe dust and vapors; mix stains in a vented hood.
• Avoid contaminating other things with the chemicals being used.
• Read all labels on containers and follow the prescribed safety measures.
• Be careful when handling stained gels and disposing of used staining solutions.
• Thoroughly clean up any spills on your chemical table.
• Wash your hands after each handling.
• Do not keep food in a refrigerator together with chemicals.

REFERENCES

Methods of Detection of Specific Enzymes


