ELECTRON MICROSCOPY AND CYTOCHEMISTRY

Proceedings of the second International Symposium, Drienerlo, The Netherlands, June 25-29, 1973

Editors:

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W.Th. Daems

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PREFACE

The Drienerlo Symposium on Electron Microscopy and Cytochemistry was held from June 25th to 30th, 1973, in The Netherlands. This was the fourth in a series held alternately in England and The Netherlands. The first conference was held in Oxford in 1962, and its Proceedings were published as Parts 3 and 4 of Volume 81 of the Journal of the Royal Microscopical Society. The next symposium took place in 1966 in Leiden; abstracts were published in the *Journal of Histochemistry and Cytochemistry,* Vol. 14, 739 (1966). The third meeting was held in London in 1968.

This book, which is intended to give the reader an impression of the present level of the cytochemical techniques used in electron-microscopy, contains the papers given at the Drienerlo Symposium and covers the various approaches applied within the field of electron-microscopical cytochemistry.

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ENZYME CYTOCHEMISTRY

Electron microscopy and Cytochemistry, eds. E. Wisse, W.Th. Daems, I. Molenaar and P. van Duijn.

FUNDAMENTAL ASPECTS OF ENZYME CYTOCHEMISTRY

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SUMMARY

A review is given of the factors that influence the accurate cytochemical localization of enzymes. Theory and practice of fixation procedures are discussed with special regard to the use of fixatives hitherto applied only in immunocytochemistry and to the possibilities of applying the principle of substrate protection. A scheme of the several phases of cytochemical enzyme procedures whose outcome is influenced by the reaction rates of chemical conversions and by the effect of diffusion processes is given. Basic principles of methods based on metal salt reactions are compared with those based on organic dye precipitation reactions. Diffusion coefficients, reaction rate constants of the components of the substrate turnover and the trapping reactions, spot size of the localization site and concentration as well as turnover number of the enzyme involved, are among the factors that determine the final results of the procedure. Study of the influence of variations in fixation and incubation procedures on the reaction as it occurs in the object in combination with studies on models, especially those employing enzymes immobilized in spheres or films, seems necessary to promote further progress of the field.

INTRODUCTION

Since the pioneer-work of Gomori and Takamatsu, many procedures to localize enzyme activities *in situ* have been described.[1] Enzymes can be localized cytochemically, due to their properties as antigens, by immunohistochemical procedures[2] or by autoradiographic methods based on their reactions with labeled inhibitors.[3] However, most of the methods make use of the catalytic activity of enzymes on substrates, which also is the basis of their biochemical characterization.

For enzyme localization at the ultrastructural level, this means that the incubation media should be composed such that substrate conversion will lead to an insoluble, electron-dense, and recognizable final product that is formed as close as possible to the original (*in vivo*) sites of the enzyme molecules. To prevent loss or displacement of the enzyme molecules from their *in vivo* positions and at the same time maintain a recognizable morphology during the incubation procedure, fixation is usually necessary.

In the following, some fundamental aspects of fixation procedures and the requirements of suitable incubation media in terms of their potentials for accurate localization and quantitation of the local enzymatic activities will be discussed. The accuracy of the final results can be influenced by the effect of each separate factor in the sequence of events from tissue preparation and fixation through incubation to observation of the final photographs. For the purposes of the present analysis, several factors involved in fixation and incubation will be discussed separately.

FIXATION

a) Objectives of fixation

A fixation procedure for enzyme cytochemistry should be designed to achieve the following aims as much as possible. It should preserve the morphology of the object adequately, it should stop metabolism so that autolysis is prevented and that the fixed cell and cell organelle skeletons can withstand the incubation procedures that follow. Fixation should also lead to a breakdown of membrane permeability barriers present *in vivo*, such that transmembranic diffusion - at least of low molecular weight substrates and trapping agents - becomes relatively unhampered.

It is becoming more and more clear that aldehyde fixation, especially in the initial phases, does not rapidly lead to this condition, which means that the tonicity of the compounds in the fixation medium that are (temporarily) osmotically active is of importance.[4,5] Finally, an ideal fixation procedure should anchor the relevant enzyme molecules to the cell structure, preferably by a covalent band, without much decrease in their enzymatic activity. In practice it is found that, depending on the type of enzyme and on fixation conditions, aldehyde fixation can result in 90% to 25% inactivation.[6]

b) Theory and practice of fixation

The activity of an enzyme depends on the preservation of the steric conformation at its active site(s) and the presence of co-enzymes. If we define denaturation of a protein as a (gradual) loss of quaternary, tertiary, or secondary structure, it is clear that an enzyme can be partially denaturated but still fully active enzymatically (Fig. 1).

Theoretically, therefore it is not unlikely that for a particular enzyme fixation agents and conditions can be found that, even if they denature the molecule to some extent, preserve its original activity or at least a part of it. The details of how a cross-linking agent will react with a particular enzyme and its influence on the steric conformation at the active site, however, remains largely unpredictable. This despite considerable recent progress in protein

chemistry and even for enzymes of which the molecular structure is completely known.

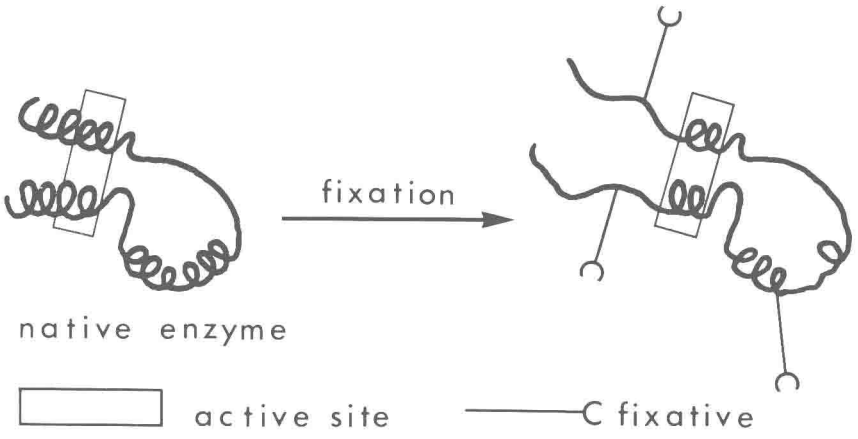

Fig. 1: Theoretical scheme of enzyme fixation. Fixation leads to modification of the original structure of the enzyme molecules: provided that during the fixation procedure the conformation of the active site (sometimes influenced by more remote parts of the molecule) remains relatively intact, loss of conformation (denaturation) caused by the fixation conditions in other regions of the molecule can be compatible with retention of (part of) the enzymatic activity.

It must therefore be stressed that there is still much to expect from trial and error experiments with new fixatives and fixation conditions. The type, ionic strength, and pH of buffers, the presence of neutral salts or hydrophylic compounds such as sucrose and dimethyl sulfoxide, the presence of reducing or oxidative compounds or of heavy metal ions, all can be factors that may prove to be useful for a particular enzyme even if detrimental for the activity of others.

Since there is a strong analogy between the conditions maintaining an active site of an enzyme and that of an immunologically active protein-antigen, enzyme-cytochemistry can learn from experience gained in immunocytochemistry and vice versa. In this respect it may be pointed out that fixatives such as carbodiimide,[7] cyanuric chloride,[8] and dimethylsuberimidate[9,10] successfully used in immunocytochemistry, have so far apparently not been applied for fixation of tissues with the aim of localizing enzymes.

Another field of research from which ideas for new fixatives could be adapted for enzyme cytochemistry are biochemical studies that use cross-linking agents to obtain more information about the steric conformation of reactive groups in proteins. Such agents have been described in great variety,[11,12] and some of

them might prove to be suitable fixatives for particular enzymes. On the other hand, fixation media that differentially inactivate one or more of a family of isoenzymes which otherwise are indistinguishable because they have similar substrate specificity,[13] can be of advantage.

c) Substrate protection

In enzymology it has been known for a long time that an enzyme can be protected to some extent against denaturing influences, such as increase in temperature, by the presence of a substrate or a reversible inhibitor. It was found as early as in the last century that sucrose protects invertase to some extent against heat inactivation.[14] Since then, many examples of this effect have become known for both substrates[15] and inhibitors.[16]

Interaction of substrate or inhibitor with the enzymatic site apparently stabilizes the conformation of the enzyme in this region. One could hope that the fixation of enzymes in the presence of a substrate or inhibitor could prevent the fixative from reacting directly with atomic groups in the site or stabilize the conformation of the site in such a way that greater part of its activity remained intact.

This principle of substrate protection (Fig. 2) has been applied successfully in enzyme cytochemistry to the isoenzymes of aspartate aminotransferase.

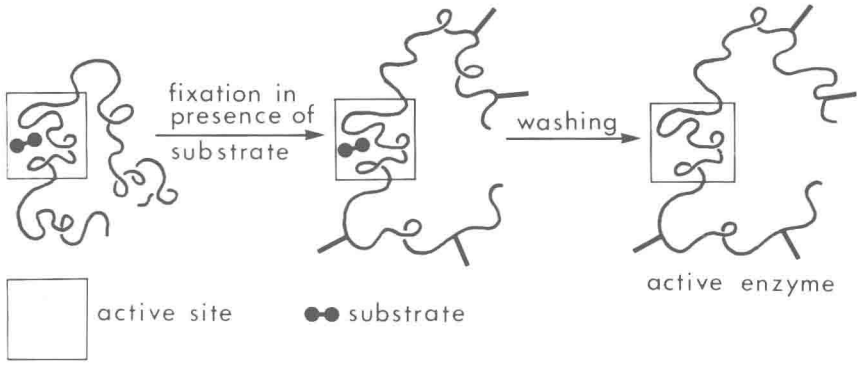

Fig. 2: Active site of the enzyme is protected during the fixation procedure by the presence of substrate or reversible inhibitor.

Fixation with glutaraldehyde or formaldehyde in the presence of ketoglutarate, one of the substrates of the enzyme, decreased the inactivation of the enzyme[17] by the fixatives. The favourable effect of adding ATP to the fixative for demonstration of ATPase activity had already been described independently[18] and has been applied to fix a muscle ATPase.[19,20]

It can be expected that further application of this principle in enzyme cytochemistry might be successful, since for most enzymes a whole range of substrates and reversible inhibitors have been described. Given the sensitivity of protein structure to its chemical environment in terms of pH, salts, etc., it again must be stressed that failure to obtain protection by applying the principle in a certain medium with a certain fixative should not discourage trial and error experiments under other conditions.

d) Quantitative model studies

The effect of a variety of fixation procedures on the accurate cytochemical localization and quantitation of enzymes must finally be judged on the basis of the localization and amount of electron-dense product produced by the action of the enzyme within the biological object. Without detracting from the value of attempting to draw conclusions concerning to the real situation of the enzyme *in vivo* from visual observation or even densitometric quantitation of the pictures obtained, it is clear that both fixation and staining are very complex phenomena, in which a great number of unknown or only partially known factors are involved. The basing of conclusions solely on the final outcome of such complex procedures is a particularly dangerous undertaking. Situations in which a given variation in the procedure is favourable for one step and unfavourable for another, could remain unrecognized if possibilities to study both parameters independently and quantitatively were lacking. Exclusive adherence to such an approach, which is very useful at the start, could in the long run put an end to further development in the field.

A number of quantitative studies, with biochemical,[6] or cytochemical media,[21] on homogenates or sections of the biological object, have been reported. Since in these cases the complexities of the biological object still play a role, models have been developed in which with both cytochemical and biochemical media, quantitative study under more sharply defined conditions, such as the use of purified (iso)enzymes, is possible.

In this respect films of polyacrylamide, which can function as vehicle for purified enzymes preparations as well as for homogenates or for cell organelle suspension, have been used successfully.[22,23] Together with a specially developed film colorimeter (Fig. 3), this method offers an elegant system in which most of the aspects of enzyme cytochemistry can be quantitatively studied in detail, both biochemically and cytochemically. With this system the different sensitivity of the isoenzymes of aspartate aminotransferase for aldehyde fixatives and the effect of substrate protection (Fig. 4), as well as the Michaelis constants for the cytochemical media, have been determined.[17] A similar system has been used to study and improve the conditions that govern light microscopical localization and quantitation of leucocyte alkaline