# Immunoenzymatic Techniques

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Developments in Immunology Volume 18

# IMMUNOENZYMATIC TECHNIQUES

Proceedings of the Second International Symposium on Immunoenzymatic Techniques, held in Cannes, France, 16-18 March, 1983

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## Preface

This book contains the proceedings of the Second International Symposium on Immunoenzymatic Techniques, which took place in Cannes, in March 1983, eight years after the First Symposium which was organized in 1975 in Paris. Although it was possible at that time to assemble almost every researcher engaged in Immunoenzymatic Techniques and to publish his work, eight years later, there are so many developments in so many different subjects, that the Editors made the decision to select for publication only the most representative works of the current research in this field. The first part of the book - about one third of its length - is devoted to enzyme immunocytochemistry with emphasis on the intracellular penetration of labelled antibodies, use of double staining methods or monoclonal antibodies in the normal state and in various experimental or pathological conditions. The second part which is subdivided into two main chapters is devoted to the quantitative aspects of Immunoenzymatic Techniques, one chapter for new technical findings and the other for applications of these techniques to very different subjects such as biology, clinical and veterinary medicine, phytopathology and experimental diseases.

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The Editors

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## AUTHOR'S CORRIGENDUM

*p. 251 line 14*

"It is incorrectly stated that the final product, $P_2$, should increase linearly with time. Only the assay modulation increases linearly unless scavenger is added."

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# ENZYME IMMUNOCYTOCHEMISTRY

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# PENETRATION OF ENZYME-LABELLED ANTIBODIES INTO TISSUES AND CELLS : A REVIEW OF THE DIFFICULTIES

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## INTRODUCTION

Almost immediately after the discovery of the possibility of labelling antibodies with enzymes (1, 2) and of using these antibodies to locate an antigen in an organelle by electron microscopy, the difficulties raised by the intracellular penetration of labelled antibodies have been emphasized (3). In 1969, Leduc and coworkers (3) observed that even with small blocks of tissue, the penetration of such antibodies did not exceed two or three cells in depth, as checked by electron microscopy. Since then, the question of antibody penetration has been considered several times (4-8) and various procedures have been proposed to solve the difficulties involved ; in the main, these procedures consisted of using conjugates with a low molecular weight, or increasingly thin sections of tissue. However, the problems became more complicated when, for instance, it became evident to some workers that instead of using coupling agents to conjugate antibodies and enzymes as initially proposed (1, 2) thus running the risk of at least partly reducing the activity of the antibodies, it would be better to use more sophisticated techniques such as the bridge method described by Mason et al (9) or the peroxidase-antiperoxidase (PAP) system (10), which do no require a chemical reactant. Also, the problem of penetration changed when other workers proposed incubating in the antibody solution sections obtained by cryostat (3, 5) or tissue-choppers (3, 11) instead of blocks of tissue. Decisive progress was made in 1978, when membrane-permeabilizing agents were introduced (12-15). Although all the problems affecting the intracellular penetration of enzyme-labelled antibodies have not yet been solved, the degree of penetration has greatly improved.

The aim of this review is to list and discuss the different procedures proposed to facilitate the penetration of enzyme-labelled antibodies into the cells. Since it is far easier to demonstrate the presence of an antigen on

the surface of a cell than inside it, the present report will deal exclusively in the antigens located inside the cell.

One prerequisite is the ability to judge whether or not antibody penetration is satisfactory. There is no theoretical criterion by which to answer this question. Optical microscopy can provide arguments permitting appreciation of a new method. For instance, if the number of cells labelled increases significantly, the penetration can be considered as ameliorated. However, optical microscopy is insufficient because five micron-thick paraffin-embedded sections cannot be cut transversely. The situation is more complicated when there is no increase in the number of cells but an augmentation in the products of the histochemical reaction indicating the presence of the antigen. Several procedures, such as the indirect method (16), or the PAP or other bridge methods (9, 10, 17) have been proposed to intensify the reaction. Even when optical microscopy shows that these methods are effective, this does not necessarily imply an improvement in intracellular penetration (7, 18). Semithin transverse sections, prepared as for electron microscopy, have occasionally been used (19, 20), but it is only with ultrathin transverse sections that one can be sure an enzyme-labelled antibody has correctly penetrated into tissues or cells (18, 21). Transverse sections are also necessary for cell cultures, but obviously not for isolated cells.

The following part will be devided into two paragraphs respectively dealing with biochemical and morphological procedures. In the first, we shall discuss the means proposed to obtain good penetration conjugates, and in the second, the methods used to prepare the tissues in such a way that they become permeable to labelled antibodies. It should be stressed that, even now, satisfactory penetration does not involve one factor only, but is obtained when several factors are brought under control.

## BIOCHEMICAL PROCEDURES

Biochemical procedures are based on the assumption that intracellular penetration of labelled antibodies depends on the molecular weight of the conjugate : the lower this weight, the better the penetration. Although this proposition is purely theoretical, several attempts have been made to reduce the weight of the conjugate, when the direct method is used.

The first way in which this was done was by preparing Fab fragments from IgG molecules. Fab fragments were introduced in immunoenzymatic techniques in 1971 by Avrameas and Ternynck (22) and by others (11). These fragments are labelled with peroxidase by techniques similar to those used for complete

antibodies (22). Sometimes Fab fragments are remplaced by Fab' fragments (8). As the molecular weight of these two fragments is almost identical, the choice of one rather than the other obviously depends on the possibilities of the laboratory. The results obtained with these fragments are not absolutely convincing, since they are often used in conjunction with other means, also known to facilitate penetration (11, 22). However, when we recently used electron microscopy to compare intracellular penetration by labelled complete antibodies and by Fab fragments (18), we observed that with Fab fragments the labelled organelles were seen often throughout the entire thickness of the section, while with complete antibodies the labelled organelles were generally confined to the surface of the section and were rarely visible inside it (Figure 1a and b). Fab or Fab' fragments are also used with the indirect or PAP methods ; it seems that even here the intracellular penetration is better with fragments than with complete antibodies (23).

The second way in which attempts have been made to reduce the molecular weight of the conjugates is by using enzymes with a weight lower than that of peroxidase. However, this field of investigation is limited, since the enzymes used in immunoenzymatic techniques, have a higher molecular weight than that of peroxidase (40,000) ; for instance, the respective molecular weights of glucose oxidase and alkaline phosphatase are 185,000 (24) and 80,000 (3). Two attempts based this principle were nevertheless made, one with cytochrome c whose molecular weight is 12,500 (11) and the other with a fragment of this cytochrome, a heme-octapeptide with a molecular weight of 1,550 (25, 26). However, these experiments are of limited interest because both agents (11, 25) were used together with Fab fragments, and the part played by the enzyme in the penetration was not decisive. In addition, the difficulty in obtaining the heme-octapeptide meant that it was only used in a few cases (25, 26). Another point about cytochrome c or heme-octapeptide is that in terms of cytochemistry, their enzyme activity is probably lower than that of peroxidase itself since their use requires more diaminobenzidine (11), suggesting that any advantage obtained as regards penetration is lost during the cytochemical reaction, which has to be prolonged, thus involving the risk of increased artifacts.

Also connected with the molecular weight of the conjugate is the technique by which the enzyme and antibody are linked. The following, are the bifunctional reactants most often used for this purpose :

- difluorodinitrodiphenyl sulfone (27)
- glutaraldehyde (28)


Fig. 1. Estimation of conjugate penetration in liver tissue by electron microscopic examination of transversely cut cryostat sections. Localization of rat fibrinogen (immersion fixation with paraformaldehyde).
a) Using Fab fragments labelled with peroxidase, fibrinogen can be detected in the endoplasmic reticulum (ER) either throughout the entire section thickness (left-handed hepatocyte), or in the superficial area only (right-handed hepatocyte). x 9 000.
b) Using antibodies labelled with peroxidase (direct method), fibrinogen is detectable only in the superficial area of the section. Similar results are obtained with the indirect or PAP methods. x 8 000.