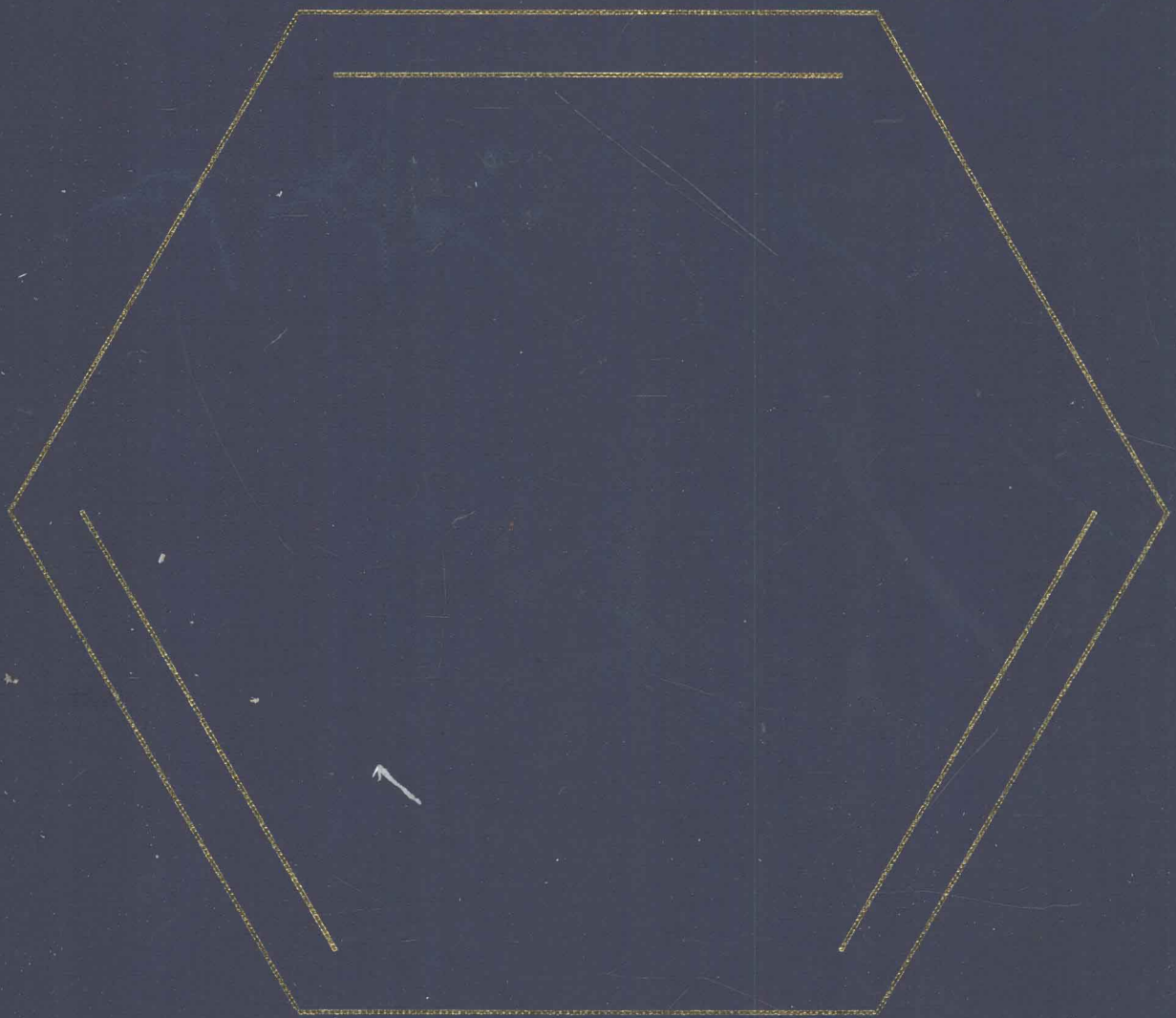
Histochemistry

VOLUME THREE

Edited by

PETER J. STOWARD

A. G. EVERSON PEARSE



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Volume 3 Enzyme histochemistry

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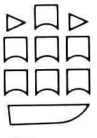

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Preface

This volume, child of many prayers (ours) and of many talents (others), completes for 'Histochemistry, Theoretical & Applied' a span of almost 40 years.

When the first volume of the work appeared in 1953, Enzyme Histochemistry was the rising star of a resurgent discipline, resurgent only after seven decades in a wilderness made for it by Johann Friedrich Miescher's 'Guild of Dyers', namely the histological fraternity.

For at least the next two decades it formed the advancing front of the whole discipline, and particularly for the field of Diagnostic Pathology. Here it promised, and made, considerable advances in the search for its 'Philosopher's Stone', distinction between a benign cell and it's malignant counterpart.

By the early 1980s the relative importance of Enzyme Histochemistry had begun to decline, especially with respect to the younger disciplines of Immunocytochemistry and Hybridocytochemistry. Nevertheless it continues to form a significant and far from static branch of the whole subject. For example, there have been enormous advances in quantification of enzyme activity in situ, and a far greater understanding of the fundamental mechanisms underlying many histochemical reactions. The size and complexity of the text provided in this volume is a direct response to the creation, since publication of the previous relevant volume in 1972, of a mass of new technology. This accompanies an ever widening range of histochemically demonstrable enzymes and enzyme groups.

We feel no need to apologise here for maintaining the principle enshrined in previous editions, of complete and up-to-date biochemical and histochemical coverage for each enzyme described.

We wish to acknowledge our great indebtedness to all those who have contributed to the final volume of the 4th Edition. We trust that they will feel that their time of waiting, whether patient or impatient, has been rewarded, and that this volume will stimulate and foster both the understanding and the application of the valuable techniques of Enzyme Histochemistry.

A.G.E.P., P.J.S.
Exeter and Dundee
1991

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Enzymes reviewed

The localisation and function of the following enzymes are described in this Volume.

Enzyme	*Contributors*
Acetyl-CoA carboxylase	PJS
Acetylcholinesterase	CO, PRL
β-N-Acetylgalactosaminidase	RG, ZL, PJS
N-Acetyl-β-glucosaminidase	RG, ZL, PJS
β-N-Acetylhexosaminidase	RG, ZL, PJS
Acetylserotonin methyltransferase	PRL, PJS
Acid phosphatase	MB, AV, PJS
Acrosin	PJS
Acyl-CoA oxidase	PJS
Acylglycerol palmitoyltransferase	PRL
Adenylate cyclase	PJS
Alcohol dehydrogenase	PJS, PRL
Alcohol oxidase	PJS
Aldehyde dehydrogenases	PJS, EW
Aldehyde oxidase	PJS
Aldehyde reductase	PJS
Aldose reductase	PJS
Alkaline phosphatase	MB, AV
D-Amino-acid oxidase	PJS, JMR, MJK, PRL
4-Aminobutyrate aminotransferase	PRL
Aminopeptidase A	ZL, RG, PJS
Amylases (α- and β-)	RG, ZL, PJS
Apyrase	PJS
α-L-Arabinofuranosidase	RG, ZL, PJS
Arginine aminopeptidase	ZL, RG, PJS
Aromatic L-amino-acid decarboxylase	PJS
Arylamine acetyltransferase	PRL
Arylsulphatase	PJS

Enzyme	*Contributors*
Aspartate aminotransferase	PRL
Aspartate carbamoyl transferase	PRL
ATPases	JAF
Benzaldehyde dehydrogenase	PJS
Benzylamine oxidase	PJS, PRL
Betaine-aldehyde dehydrogenase	FW
Calcidiol 1-monooxygenase	PJS
Carbamoyl-phosphate synthase	PJS
Carbonic anhydrase	CO, PJS
Carbonyl reductase	PJS
Carnitine acetyltransferase	PRL
Catalase	WD, SA, HDF
Catechol methyltransferase	PJS
Catechol oxidase	PJS
Cathepsin B	ZL, RG, PJS
Cathepsin D	ZL, RG, PJS
Cathepsin G	ZL, RG, PJS
Cathepsin H	ZL, RG, PJS
Cathepsin L	ZL, RG, PJS
Cellulase	RG, ZL, PJS
Cellulose 1,4-β-cellobiosidase	RG, ZL, PJS
Chitinase	RG, ZL, PJS
Cholesterol esterase	CO
Cholesterol oxidase	PJS, JMR, MJK
Choline acetyltransferase	PRL, PJS
Choline dehydrogenase	FW
Cholinesterases	CO, PRL
Chymase	ZL, RG, PJS
Collagenases	ZL, RG, PJS
Creatine kinase	PRL

Enzyme	*Contributors*
IMP dehydrogenase	PJS
Indoleamine 2,3-dioxygenase	PJS
Inorganic pyrophosphatase	MB, AV
Isocitrate dehydrogenases	AEFHM
Ketohexokinase	PJS
Lactase	RG, ZL, PJS
Lactate dehydrogenase	AEFHM, PRL, PJS
Leucocyte elastase	ZL, RG, PJS
Lysophospholipase	CO
Lysozyme	RG, ZL, PJS
Lysyl oxidase	PJS
Malate dehydrogenases	AEFHM, PJS
Malate synthase	PJS
Mannosidase	RG, ZL, PJS
Membrane metallo-endopeptidase	ZL, RG, PJS
Microsomal aminopeptidase	ZL, RG, PJS
Monoamine oxidase	PJS, JMR, MJK, PRL
NAD(P)H oxidases	PJS, JMR, MJK
NAD^+ kinase	PJS
NADH dehydrogenases	PJS
NADH-ferrihemoprotein reductase	PJS
NADP(H) phosphohydrolases	MB, AV, PJS
NADPH dehydrogenases	PJS
Nicotinamide-nucleotide adenyltransferase	PRL
Nicotinate-nucleotide pyrophosphorylase	PJS
Nitrate reductase	PJS
Non-specific esterases	CO, PJS, PRL
Nucleoside-diphosphatase	PJS, MB, AV
Nucleoside-triphosphatase	MB, AV
3′-Nucleotidase	MB, AV
5′-Nucleotidase	MB, AV
Oligo-1,6-glucosidase	RG, ZL, PJS
Ornithine aminotransferase	PJS
Ornithine carbamoyltransferase	PRL
Ornithine decarboxylase	PJS
Peroxidases	WD, SA, HDF
Phenolases	PJS
Phenylethanolamine N-methyltransferase	PJS
Phospho*enol*pyruvate carboxylase	PJS
Phospho*enol*pyruvate carboxykinase	PJS
6-Phosphofructokinase	PRL
Phosphoglucomutase	PRL, PJS
Phosphogluconate dehydrogenase	ES, CJFVN, PJS
Phosphorylase	PRL
Plasmin	ZL, RG, PJS
Plasminogen activator	ZL, RG, PJS
Platelet peroxidase	PJS
Polyphenol oxidases	PJS, JMR, MJK
Prolyl hydroxylase	PJS
Prostacyclin synthase	PJS
Prostaglandin synthase	PJS
Protochlorophyllide reductase	PJS
Protein-disulphide reductase	PJS
Purine-nucleoside phosphorylase	PRL
Pyruvate dehydrogenase complex	PJS
Pyruvate kinase	PRL, PJS
Renin	ZL, RG, PJS
Retinol dehydrogenase	PJS
Ribose-5-phosphate isomerase	PJS
Ribulose bisphosphate carboxylase	PJS
Secondary alcohol dehydrogenase	PJS
Serine-pyruvate aminotransferase	PJS
Sialidase	RG, ZL, PJS
Submandibular protease A	ZL, RG, PJS
Succinate dehydrogenases	PJS
Succinate-semialdehyde dehydrogenase	PJS
Sucrose-α-glucosidase	RG, ZL, PJS
Sulphinoalanine decarboxylase	PJS
Superoxide dismutase	PJS
Terminal deoxynucleotidyl transferase	PJS
Tetrametaphosphatase	PJS
Thiamine monophosphatase	MB, AV
Thiamine pyrophosphatase	MB, AV, PJS
Thioglucosidase	RG, ZL, PJS
Thioredoxin reductase	PJS
Thiosulphate sulphurtransferase	PJS, PRL
Tissue kallikrein	ZL, RG, PJS

Contents

21. Principles of oxidoreductase histochemistry

P. J. Stoward F. P. Altman E. Seidler

The commonest method of localizing oxidoreductases histochemically is based on one simple principle — the reduction, by the electrons released in the reaction catalysed by the enzyme, of a colourless, soluble tetrazolium salt to an intensely-coloured, highly insoluble derivative called a formazan. This is an oversimplification of what actually occurs. In 1972, when the previous edition was published, it was not widely appreciated how serious this oversimplification was. Several techniques recommended at that time have since been shown to be grossly inadequate and to give false results. For example, it was not realised that soluble enzymes diffused very rapidly out of sections into aqueous incubation media; consequently, the amount and distribution of the final reaction product (i.e. staining) subsequently seen in sections bore little relation to the original activity of the enzyme in situ. Unfortunately, many workers have continued to use these techniques despite the wealth of evidence of their shortcomings published in the two decades since 1970.

This chapter is, therefore, devoted to the principles on which the modern histochemistry of oxidoreductases is based. It is particularly concerned with the special factors that should be taken into account in order to obtain a reliable, accurate and specific localization. The most important concerns are; the reaction mechanisms underlying oxido-reductions, the characteristics of tetrazolium salts, intermediate electron acceptors and cofactors; and the suppression of the leakage of enzymes from unfixed cells and tissues during incubation. The account that follows is based partly on Chapter 20 of the last edition, but to a greater extent on texts by Altman (1972, 1976a), Wohlrab et al, (1979), Van Noorden (1984) and Stoward (1991).

OXIDOREDUCTASE-CATALYSED REACTIONS

Oxidoreductases (EC class 1) catalyse reactions between *two* substrates in which the principle substrate ($S.H_2$), called the donor, is oxidized by the other, the acceptor (A). Such reactions are generally written in the form

$$\underset{\text{Donor}}{S.H_2} + \underset{\text{Acceptor}}{A} \longrightarrow S + AH_2$$

The donor is not always dehydrogenated as shown in this equation, but when it is, the catalysing enzyme is known as a dehydrogenase. However, two electrons are always removed from the donor. The electrons are transferred to, and thus accepted by, either the enzyme itself or separate molecules such as coenzymes or special proteins within the cell. The vast majority of dehydrogenases fall into two groups, *flavin-containing dehydrogenases* and *coenzyme-linked (or pyridine-linked) dehydrogenases*.

The flavin-containing dehydrogenases contain electron acceptors bound to the enzyme molecule itself. These acceptors are known as prosthetic groups, and are either a flavin adenine nucleotide (FAD) or a flavin mononucleotide. Succinate dehydrogenase belongs to this group.

The second group of dehydrogenases have no integrally-bound acceptors, and function only in the presence of separate electron acceptors or carriers known as coenzymes, usually NAD^+ or $NADP^+$.

Apart from these two groups, there is a small group of oxidoreductases which require other

acceptors, of which the most important are hydrogen peroxide (the peroxidases) and molecular oxygen (the oxidases).

The initial products of any biochemical reaction catalysed by a dehydrogenase are either a reduced flavin (for example, $FADH_2$ in the case of succinate dehydrogenase) or a reduced coenzyme (for example, NADH in the case of NAD^+-dependent lactate dehydrogenase) plus, in both instances, the appropriate oxidized substrate. The reduced acceptors normally must be immediately re-oxidized to allow the enzymes concerned to go on functioning. Cells and tissues have a number of pathways to achieve this re-oxidation, the most well-defined ones being the respiratory chain in mitochondria and the cytochrome P_{450} system. They are discussed in detail later in connection with their relationships to the reduction of tetrazolium salts.

Thus, oxidation and reduction reactions catalysed by oxidoreductases do not necessarily involve oxygen and hydrogen. The fundamental event is the exchange of electrons; the passage or transfer of hydrogen or oxygen atoms is incidental. Nonetheless, it is often more convenient to visualize oxido-reduction reactions as 'hydrogen transport' rather than as 'electron transfer'.

PRINCIPLES UNDERLYING THE HISTOCHEMICAL LOCALIZATION OF DEHYDROGENASES

In most techniques, cell smears or sections are incubated in a suitably buffered medium containing:

1. the substrate of the enzyme to be localized
2. any necessary cofactor (NAD^+ or $NADP^+$)
3. an intermediate electron acceptor
4. a tetrazolium salt or some other final electron acceptor.

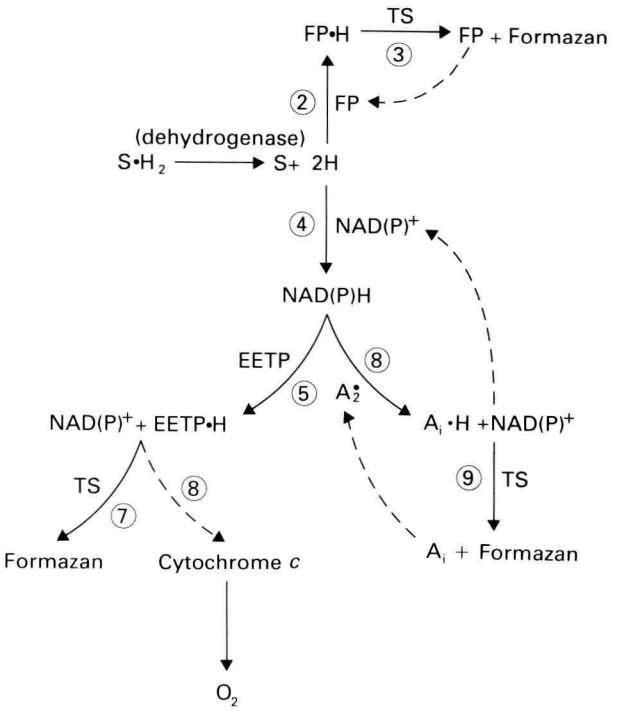
In addition, if the dehydrogenase is soluble, an inert tissue stabilizer, such as suitable grade of polyvinyl alcohol (PVA), should be dissolved in the medium to prevent the leakage of the enzyme from the section into the medium during incubation.

The dehydrogenase being localized oxidises the substrate ($S{\cdot}H_2$) in the medium to a reduced intermediate reaction product (S), releasing electrons and hydrogen which pass either to the flavin component (FP) of the enzyme molecule or, if a coenzyme-linked dehydrogenase is being demonstrated, to exogenous oxidised coenzymes (NAD^+ or $NADP^+$) in the substrate medium. These are shown in Fig. 21.1 as reactions 2 and 4.

When a flavin-linked dehydrogenase, such as succinate dehydrogenase, is being localized, the reduced flavoprotein ($FADH_2$ or FADH) usually has a sufficiently high redox potential to reduce the tetrazolium salt in the medium to form an insoluble formazan, which then becomes deposited on the subcellular site of the enzyme.

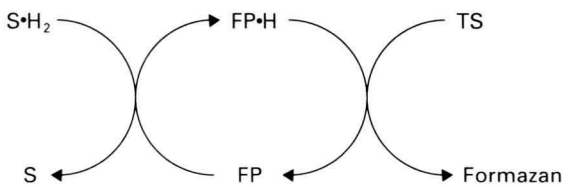
In contrast, the reaction paths followed during the localization of a coenzyme-linked dehydrogenase are more complex. The reduced coenzymes (NADH or NADPH) formed in reaction 4 (Fig. 21.1) are, unless steps are taken to prevent it, almost immediately oxidized by the *endogenous electron-transfer protein* (EETP) systems present in most cells. The reducing equivalents thus formed are either passed onto endogenous acceptors, such as cytochrome *c* (reaction 7), or onto exogenous acceptors such as molecular oxygen (reaction 8), or they reduce the tetrazolium salt in the substrate medium. If reaction 5 occurs to a significant extent, the insoluble formazan arising from the reduction of the tetrazolium salt is deposited on the subcellular site of the endogenous electron-transfer system, and not on the site of the enzyme. Thus, the resulting localization is 'false'.

In order to achieve a specific localization of a coenzyme-linked dehydrogenase, the endogenous electron-transfer systems of the tissue under study must be bypassed. This is achieved by adding an intermediate electron acceptor (A_i), such as phenazine methosulphate, to the substrate medium. The acceptor is reduced by the reducing equivalents (i.e. the electrons and protons released by the enzyme) to form a reduced intermediate which, in turn, immediately reduces the tetrazolium salt in the substrate medium. This results, in theory at least, in the deposition of an insoluble formazan on the subcellular site of the initial substrate-specific dehydrogenase (reactions 8 and 9 in Fig. 21.1). In this situation, the



Alternatively the above reaction sequences may be written in the form normally drawn in biochemistry textbooks for coupled oxidation-reduction reactions as follows

For flavin-containing enzymes:



For pyridine-linked dehydrogenases:

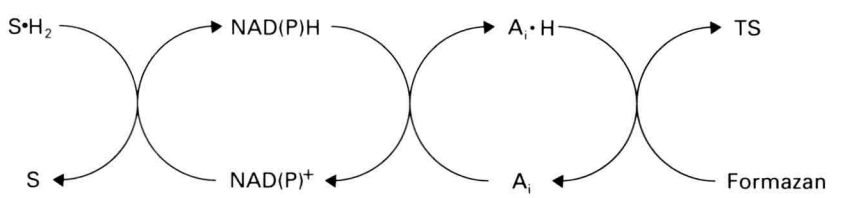


Fig. 21.1 Reaction sequences involved in the localization of coenzyme-linked and flavoprotein-linked dehydrogenases.