Electron Microscopy of Enzymes

Volume 2

Principles and Methods

EDITED BY **M.A. HAYAT**

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Electron Microscopy of Enzymes

Principles and Methods

It is a pleasure to dedicate this volume to

Alex B. Novikoff and Arnold M. Seligman

Preface

This is the second volume in the series on the principles and methods employed for studying enzymatic activity. It is encouraging to know that the first volume has been favorably accepted. This volume has developed, over the years, through the joint effort of ten distinguished author-scientists. The book contains new viewpoints with particular regard for current problems.

It is my impression that this volume will fulfill its purpose: to provide an understanding of the usefulness, limitations, and potential of the preparatory procedures used for studying enzymatic activity. I hope that it may prove to arouse more interest in the importance and problems of electron cytochemistry, and to motivate a deeper and refined study of enzymatic activity.

It is a pleasure to acknowledge the cooperation shown by Mrs. Alberta Gordon of Van Nostrand Reinhold Company.

M. A. HAYAT

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Hemoproteins

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INTRODUCTION

The application of histochemical methods to the study of hemoproteins was initiated at the turn of the century when Fischel (1910) introduced benzidine as a substrate and demonstrated a thermolabile peroxidase activity in the granules of eosinophilic and neutrophilic leukocytes. The procedure was subsequently modified by Graham (1918), who also suggested the use of α-naphthol. In later studies, additional substrates were proposed as indicators in peroxide-peroxidase systems (Pearse, 1961). These include orthophenylenediamine, the so-called leuko dyes, and 3-amino-9-ethyl-carbazole (Graham *et al.,* 1965), introduced originally by Burstone (1960) for the demonstration of cytochrome oxidase and aminopeptidase activities. Additional references to the earlier literature may be found in the review by Agner (1941). Despite the availability of these and other compounds, benzidine has proved to be the substrate of choice for the light microscopic demonstration of hemoproteins. However, neither benzidine nor the other substrates mentioned above are useful for electron cytochemistry.

The localization of peroxidase activity at the ultrastructural level with

benzidine as substrate was first reported by Mitsui (1960), who demonstrated reaction product in the granules of salamander leukocytes. However, the deposits obtained after incubation in benzidine media are generally of irregular size and shape, and have relatively low electron opacity (Mitsui, 1960; Graham and Karnovsky, 1966a; Goldfischer and Essner, 1969). This may necessitate densitometry (Mitsui, 1960) to confirm the reaction or inordinately long incubations to accumulate sufficient end product. For these reasons, the benzidine procedure has not been extensively used for the electron microscopic study of peroxidase or other hemoproteins.

Several substituted benzidines such as *o*-tolidine, 2,4-diaminofluorene and 2,7-diaminobenzidine have been suggested (Ornstein, 1968). These substrates yield oxidation products that couple rapidly with α-naphthol, forming extremely insoluble precipitates. Apparently, however, they have not yet been tested at the ultrastructural level.

Interest in the study of hemoproteins was revived when Graham and Karnovsky (1966a) introduced 3,3′-diaminobenzidine (DAB) (Fig. 1–1) and demonstrated its usefulness for the ultrastructural localization of peroxidase activity. Oxidized DAB is readily visualized by both light and electron microscopy. These authors also noted that *o*-dianisidine can be substituted for DAB, but that the end product lacks sufficient electron density.

The DAB procedure or one of its subsequent modifications has since been applied extensively to the localization of hemoproteins in various cells and tissues. Reaction product has been localized in structures such as endoplasmic reticulum, nuclear envelope (Fig. 1–2), Golgi saccules, and various types of granules. It has also been visualized in smaller cytoplasmic components such as pinocytosis vesicles (Fig. 1–6) and ribosomes (see discussion below). These observations are indicative of the high resolution that can be achieved with the DAB procedures. This is due in large measure to the unique properties of oxidized DAB, which may be summarized as follows: finely granular or amorphous form; insolubility in dehydration and embedding agents; high opacity due to formation of insoluble, polymeric complexes with osmium (Fig. 1–1) (Hanker *et al.*, 1967; Seligman *et al.*, 1968); and minimal diffusion under the usual conditions of incubation (however, see below, "Diffusion Artifacts").

Almost all hemoproteins containing an iron porphyrin prosthetic group display peroxidase or peroxidatic activity, and are therefore potentially demonstrable with the DAB procedures. These include the protoheme peroxidases, catalase, the nonenzyme hemoproteins such as hemoglobin, myoglobin, and cytochrome c, and certain heme-containing proteins found in lysosomes and related organelles. Not included in this list are the flavoprotein peroxidases, which contain flavin adenine dinucleotide as prosthetic

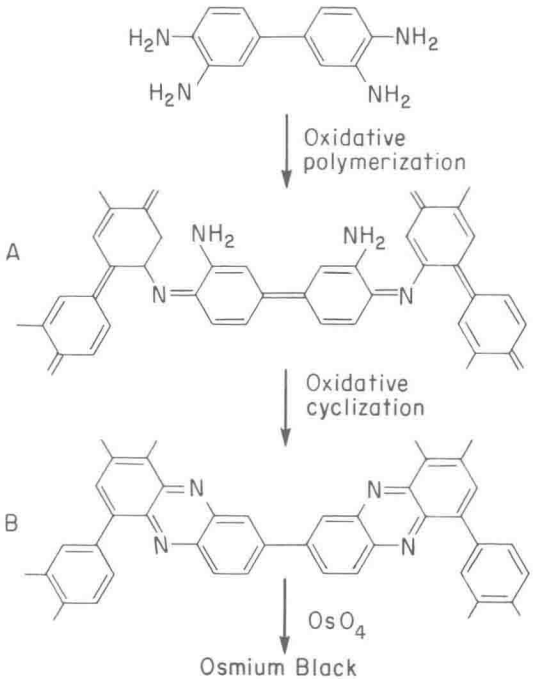

Fig. 1–1. Hypothetical formulation of the oxidative polymerization of DAB to an indamine polymer (A). This may be followed by further quinoid addition to the primary amine resulting in oxidative cyclization to a phenazine polymer (B). A. M. Seligman *et al.*, *J. Cell Biol.* **38,** 1 (1968).

group and lack hematin or metals in significant amounts. For example, glutathione peroxidase found in liver, blood, and other tissues is insensitive to cyanide and azide (Paul, 1963); it does not oxidize *p*-tolidine or guaiacol, and would therefore probably not oxidize DAB. However, such enzymes might be demonstrated by applying the methods recently developed by Hanker *et al.* (1972a and b). These authors have shown that certain transition metal compounds (e.g., cupric ferrocyanide) are capable of catalyzing the nonenzymatic oxidative polymerization of DAB to an indamine-type osmiophilic polymer, and that this principle can be exploited cytochemically for the demonstration of hydrolases and dehydrogenases.

Seligman and colleagues have studied two compounds chemically related to DAB which are oxidized in certain tissues. These are N,N′-bis (4-amino-phenyl)-1,3-xylylenediamine (BAXD) and N,N′-bis (4-amino-phenyl)-N,N′ dimethyl ethylenediamine (BED) (Seligman *et al.*, 1970; Nir and Seligman, 1971). Both compounds are oxidized by horseradish