Quantitation of Catalase Activity by Microspectrophotometry After Diaminobenzidine Staining

A. Geerts* and F. Roels

Laboratorium voor Menselijke en Vergelijkende Anatomie, Rijksuniversiteit Gent and Laboratorium voor Menselijke Anatomie, Vrije Universiteit Brussels, Laarbeeklaan 103, B-1090 Brussels, Belgium

Summary. The absorbance of the reaction product of catalase staining with diaminobenzidine is linearly proportional to enzyme activity. This is shown in semithin Epon sections of model systems containing serum albumin and catalase from bovine or guinea pig liver. Absorbance measurements were also performed on semithin sections of guinea pig liver, and from these, the activity of cytoplasmic (extraperoxisomal) catalase has been derived.

Introduction

Widespread use of the diaminobenzidine (DAB) methods for catalase (Novikoff and Goldfischer 1969; Fahimi 1969) has demonstrated differences in staining intensity in circumstances in which the reaction was performed under identical conditions. Some examples of these differences are: (i) differences in peroxisome staining between individual human biopsies of liver and kidney (Roels and Goldfischer 1979); (ii) between individual hepatocytes (Roels 1976); (iii) between hepatocytes of mouse and rat on the one hand, and those of sheep, monkey and guinea pig on the other, with respect to cytoplasmic reaction product (Roels 1976; Roels et al. 1977); (iv) between groups of cells in developing hepatoma nodules (Tsukada et al. 1979).

The present investigation has examined whether such differences in cytochemical staining may be interpreted quantitatively in terms of catalase activity. A technique was developed that permits estimation of enzyme activity in light microscopical sections. Parts of the results were reported previously (Geerts et al. 1977, 1980).

Materials and Methods

Catalase. Enzyme came from two sources: (a) purified beef liver catalase in solution, commercially available from Boehringer (n=15675). (b) prepared from male guinea pig liver as follows: under

* I.W.O.N.L. bursaal 1977–1979; present address: Laboratory for Cell Biology and Histology, Free University Brussels, Laarbeeklaan, 103, B-1090 Brussels, Belgium
anesthesia, liver was rinsed with saline through the v. portae and then homogenized in a Virtis apparatus at 35,000 turns during 1 min. The homogenization medium contained 10% sucrose, $10^{-2}$ M EDTA and 22 mM ethanol in 0.01 M imidazole/HCl buffer at pH 7.2. Centrifugation at 105,000 g during 3 h yields a supernatant that is used as the catalase source. The enzyme preparations were concentrated in a Minicon B15 concentrator (pore size: MW 15,000).

**Catalase Activity.** The catalatic activity of the enzyme solutions was determined with titanium oxysulfate according to Baudhuin et al. (1964); reaction time was 10 min at 0°C.

**Model System.** Catalase was immobilized in a bovine serum albumin (BSA) matrix, a technique introduced by Broun et al. (1973). Immobilisation is carried out by means of glutaraldehyde (Schejter and Bar-Eli 1970; Balcom et al. 1971; Ferrier et al. 1972; Broun et al. 1973; Bouin et al. 1976), which concomitantly gelifies the protein mixture. Simultaneously, the enzyme undergoes fixation, which is a necessary step for optimal cytochemical staining (Roels and Wisse 1973; Herzog and Fahimi 1974; Roels et al. 1975). 7% BSA was dissolved in 200 µl of the appropriate catalase concentrations, and subsequently 100 µl of 4.5% glutaraldehyde was added. The fixative was buffered with 0.12 M cacodylate +0.1% CaCl₂. After thorough mixing by pipetting, the solution was transferred to BEEM capsules. After gelification, which proceeds within minutes, the capsules were cut open, the protein gel sliced in 0.5 mm blocks and fixation continued up to 3 h in cold 3% glutaraldehyde. This procedure closely mimics the standard fixation by perfusion of liver as used for catalase cytochemistry (Roels et al. 1977).

In the case of highly concentrated preparations, enzyme represented up to 16% w/v; no BSA was then added and glutaraldehyde was reduced to 2%; subsequent immersion was in 3% glutaraldehyde. In order to reach the same total protein concentration in all catalase dilutions, BSA was added up to the highest protein concentration. Protein was measured according to Lowry et al. (1951).

**Guinea Pig Liver** was fixed in vivo during pentobarbital anesthesia by perfusion with glutaraldehyde, and processed as described previously (Roels et al. 1977).

**Cytochemical Staining.** The fixed protein gels of the model system were treated in a way identical to liver. 40 µm unfrozen sections were cut with the Smith-Farquhar tissue chopper, briefly rinsed in 0.1 M cacodylate buffer containing 1% CaCl₂, and transferred to the DAB medium at 45°C. The reaction was started by addition of $H₂O₂$ (0.06% final concentration) and carried out in a waterbath under vigorous shaking. The medium was always freshly prepared by dissolving 20 mg 3,3'-diaminobenzidine.4 HCl in a few drops of distilled water, and then adding 10 ml of unbuffered 0.1 M propanediol (MW 105) containing 7.5% sucrose and $10^{-3}$ M KCN. Final pH is 9.4. DAB concentrations of 0.2% and 0.05% have been compared. After incubation, the sections were thoroughly rinsed three times in 10% sucrose, and postfixed in 1% aqueous OsO₄ for 1 h. After dehydration in ethanol, they were embedded in Epon.

**Control incubations** were of two types. (a) used DAB medium in which $H₂O₂$ is destroyed by an excess of bovine catalase (Roels 1976; Roels et al. 1977); (b) chopper sections were heat inactivated in 10% sucrose at 75°C during 3 h, prior to staining in the complete DAB medium. Thermostable peroxidatic reactions (hemoglobin, cytochrome c) are not inhibited. Controls from the model system and their corresponding catalase preparations were embedded in the same block.

**Microspectrophotometry.** Epon sections of 1,2 or 4 µm were cut with an LKB Pyramitome. Variations in section thickness were evident at the naked eye but could not be eliminated effectively. As this was felt to be a major source of potential error, measurements were always performed in at least three sections; the thinnest as well as the thickest section from a series were disqualified. The sections were mounted on glass slides and absorbance was measured under oil immersion (obj. 100×1.3) with a Leitz MPV II Mikroskop-Photometer equipped with a stabilized tungsten lamp and interference filter. In a first series of experiments extinction was measured at 435 nm ± 13 nm (band width) (Streefkerk and Van der Ploeg 1976). Subsequently 450 nm was adopted as proposed by Herzog and Fahimi (1973); a poorly pronounced absorbance maximum is discernible around this wavelength (Fig. 1). In the Leitz apparatus, the measuring diaphragm is localized...