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Quantitation of Catalase Activity by Microspectrophotometry After Diaminobenzidine Staining

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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

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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 homogenisation medium contained 10% sucrose, 10^{-3} 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% $CaCl_2$. 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 glutaradehyde 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% $CaCl_2$, and transferred to the DAB medium at 45° C. The reaction was started by addition of H_2O_2 (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 OsO_4 for 1 h. After dehydration in ethanol, they were embedded in Epon.

Control incubations were of two types. (a) used DAB medium in which H_2O_2 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

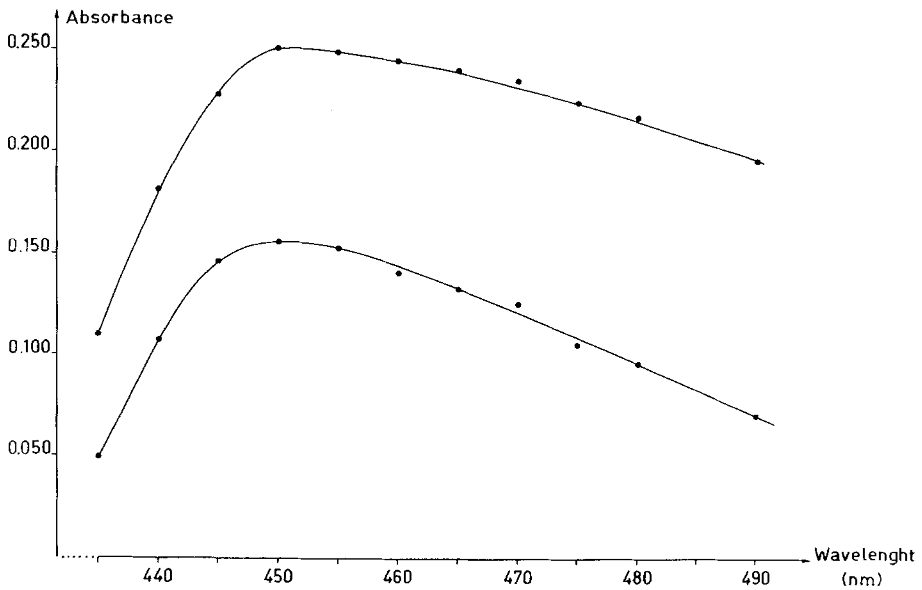


Fig. 1. Absorbance spectrum of DAB reaction product with (upper) and without postosmication (lower curve), as measured with an interference filter in 2μm Epon sections of a model system containing bovine liver catalase

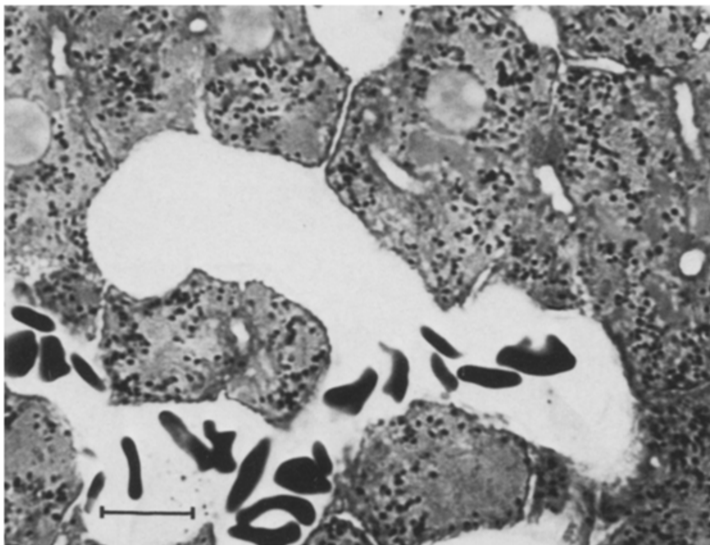


Fig. 2. 2 μm sections of guinea pig liver stained for catalase photographed under oil immersion showing cytoplasmic fields free of peroxisomes (×1,200); in such fields microspectrophotometric measurements were performed using an adjustable square diaphragm. Bar: 10 μm

between microscope and photomultiplier. For the model system a 0.5 mm diaphragm was used. At the level of the section this diaphragm allows selection of 4 µm (∅) areas. A square diaphragm of adjustable size was substituted for the measurement of cytoplasmic fields in hepatocytes. The transmitted light I_0 was determined over the Epon next to the tissue. Per model system 20 fields in at least 3 sections were measured. In guinea pig hepatocytes measurements were carried out on 2 µm sections in cytoplasmic fields free of peroxisomes (Fig. 2).

Results

Absorbance increases with incubation time, as shown in Fig. 3. The curve is not linear; after 1 h of incubation, a plateau is reached. The data suggest an exponential relationship.

Figure 4 shows four model systems with different catalase activities, stained for increasing times. The level of the extinction plateau that is reached after 1 h, is dependent upon enzyme activity.

When the extinctions after 1 h and 2 h are plotted against original enzyme activity, a linear relationship becomes evident (Fig. 5). This linear relationship was confirmed repeatedly in subsequent experiments (Fig. 6), as well as in the model system containing guinea pig liver catalase (Figs. 7 and 8). From the latter data the regression equation was calculated from 4 µm sections:

$$\text{Absorbance} = 0.00353 \times \text{activity} + 0.01560.$$

When postosmication is omitted, absorbances are still linear with enzyme activity, but lower, confirming the usefulness of Os-treatment in cases of low enzyme activity (Roels and Goldfischer 1979). When 0.05% DAB instead of 0.2% is used, all absorbances are halved.

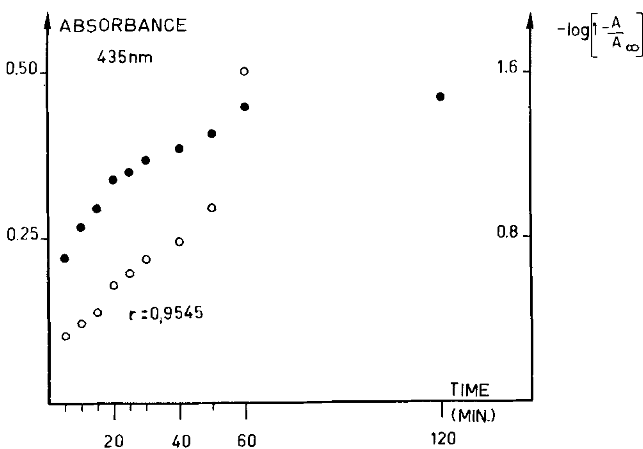


Fig. 3. Absorbance in 1 µm sections, plotted against incubation time; postosmicated. Catalase activity was 840 units (Baudhuin) per ml, i.e. approximately three times the total activity per gram of male guinea pig liver, or ten times the activity in mouse liver. Full circles: absorbance. Open circles: logarithmic function of A; a straight line is obtained with correlation coefficient $r = 0.9545$

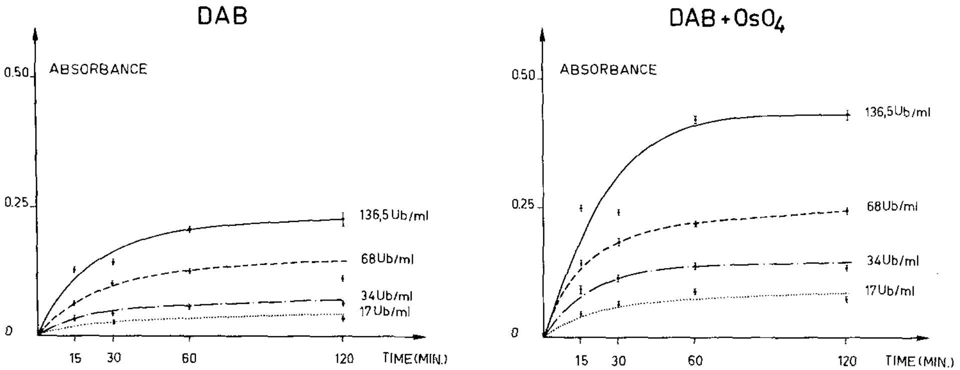


Fig. 4. Absorbance in 2 μm sections cut from model systems containing four different catalase activities. Each point is the mean of at least 20 measurements; vertical bars represent the standard error of the mean times the t-value for $p = 0.05$. Catalase came from bovine liver

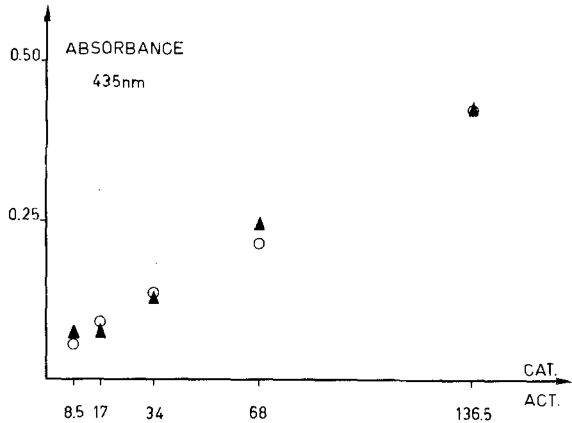


Fig. 5. Absorbance in 2 μm sections plotted against catalase activity. The lines do not pass through the origin because controls were not substracted. Circles: 1 h incubation; triangles: 2 h. Postosmicated. Correlation coefficients are $r = 0.999$ and $r = 0.997$. Cat. act.: activity in units Baudhuin per ml. Enzyme from bovine liver

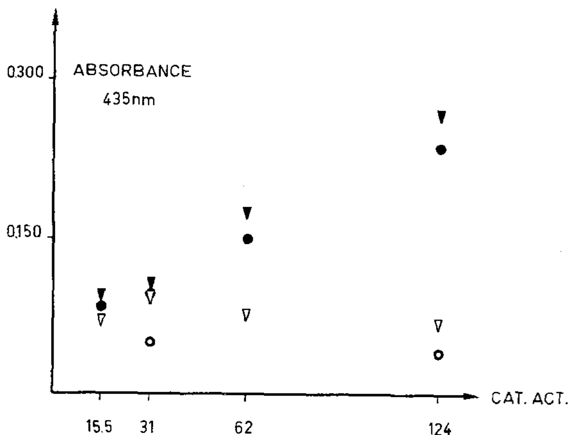
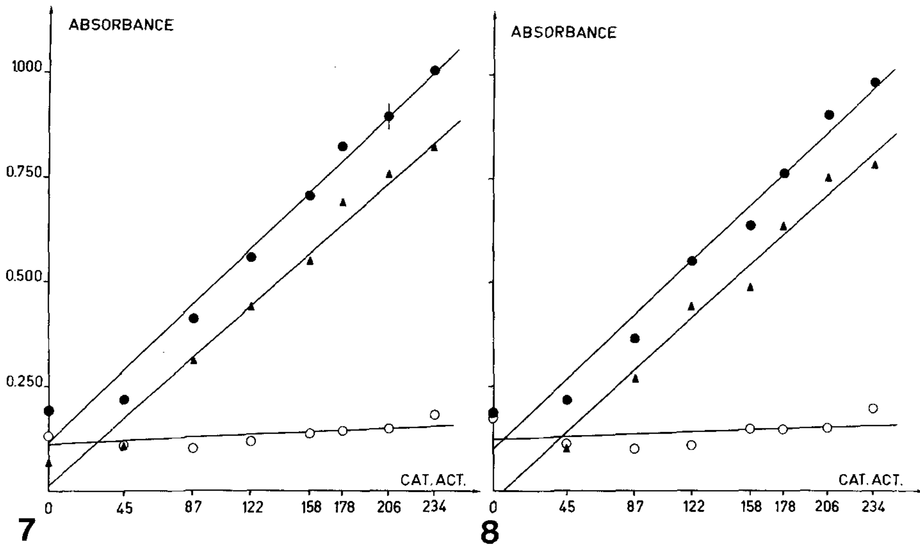


Fig. 6. Another model system from bovine liver catalase. Open symbols represent control incubations; when these values are substracted, the lines pass through zero. Circles: 1 h of incubation, triangles 2 h. Correlation coefficients are $r = 0.997$ and 0.993. Cat. act.: activities per ml, expressed in units Baudhuin



Figs. 7 and 8. Seven model systems prepared from guinea pig catalase, 4 µm sections. Each point represents the mean of 30 measurements. Closed circles: absorbance of catalase stain; open circles: controls, type b; triangles: difference. Correlation coefficients of the latter curves: $r=0.992$. Points at zero catalase activity represent model containing BSA only. Confidence limits at $p=0.05$ are smaller than the symbols, except where they are shown

Fig. 7. Incubation medium with 0.01% KCN

Fig. 8. With 0.03% KCN

Control incubation type (a) (without H_2O_2) yielded lower absorbances than control incubation type (b) (heat inactivated); preference was given to the latter in order to correct accurately for unspecific staining.

Diffusion Limitation. In models with high (800 U/ml or more) catalase activity, the center of the chopper section was less darkly stained. The same phenomenon could sometimes be observed in liver of normal guinea pigs. In order to determine whether this represented diffusion limitation of a component from the DAB medium (van Duijn et al. 1967; Thomas and Broun 1973; Barbotin and Thomas 1974) chopper sections of liver were cut at 40, 80 and 120 µm. By vigorous shaking during incubation, uniform staining was realized in the very thinnest sections, while diffusion limitation was evident in the 120 µm sections (Fig. 9). Because H_2O_2 is destroyed by the catalatic mechanism as well as by the peroxidatic staining reaction, diffusion of this reagent was expected to be the limiting factor. In order to inhibit the catalatic reaction, KCN concentration in the medium was raised to 0.03%; in model systems with high enzyme activity this resulted in more uniform staining.

Extinction Measurements in Guinea Pig Liver. Figure 10 shows an example of the absorbance frequency distribution in 60 cytoplasmic fields free of peroxisomes in 2 µm Epon sections of a normal male. The occurrence of darkly

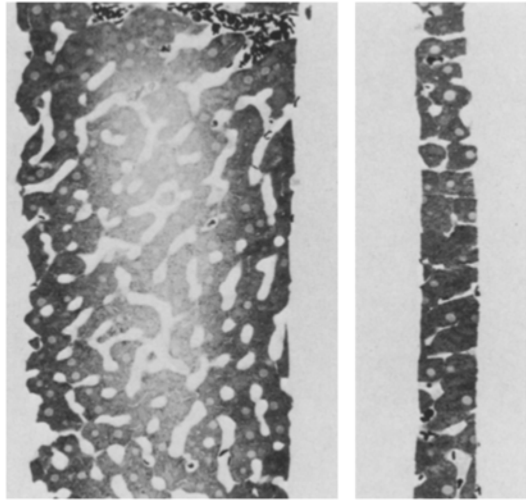


Fig. 9. Diffusion limitation in guinea pig liver, fixed by perfusion. At left, chopper section cut at 120 µm, incubated in DAB for 1 h and subsequently sectioned through its entire thickness; reduced staining is seen in the inner layers not exposed to the reagents. At right, chopper section cut at 40 µm yields uniform staining. The actual thickness clearly does not accurately correspond to the setting of the apparatus (× 160)

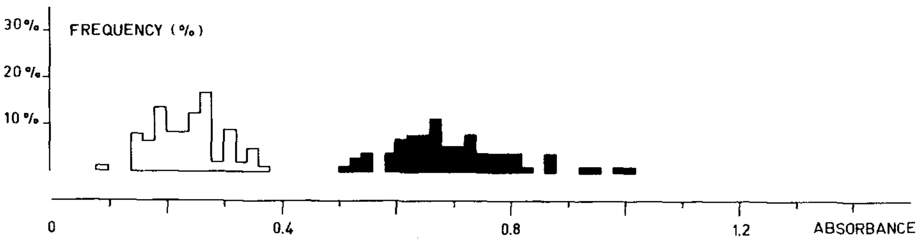


Fig. 10. Frequency distribution of absorbance in the cytoplasm of periportal hepatocytes of one animal. Black histogram: catalase stain. Open histogram: control (b) of the same liver

and less darkly stained cytoplasmic areas is reflected in the broad base of the histogram. The mean of the absorbance from 7 animals is 0.780. The mean of the extinctions in the control sections of the same livers (control b) is 0.266. The difference $0.780-0.266=0.514$ corresponds to the reaction product from catalase activity. From the regression equation corresponding to the calibration lines (Figs. 7 and 8), the mean cytoplasmic catalase activity can then be calculated to be 290 units (Baudhuin) per ml of cytoplasm (confidence limits for $p=0.05$: $\pm$ 67). From this figure the ratio of cytoplasmic catalase to total activity can be estimated as follows, bearing in mind that the morphometric data we take from the literature refer to rat liver:

1. applying a volume fraction of 0.58 between cytoplasm and total cellular volume (Blouin et al. 1977), liver parenchymal cells contain 168 units of extraperoxisomal catalase per ml.

A correction factor should be introduced if the mitochondrial volume is taken into account. Because cytoplasmic fields were measured at random, they must have contained mitochondria, and the latter do not carry catalase reaction product. As a consequence, absorbance was diminished. Maximal impact of the phenomenon would be reached if mitochondria were as numerous in the measured fields as in the rest of the cell. The volume fraction of the mitochondria

being 0.28 (Blouin et al. 1977) the measured absorbance is but 0.58/(0.28+0.58) =0.67 of the actual cytosolic absorbance. When corrected in this way, extraperoxisomal catalase actually amounts to 168/0.67=251 units per ml of parenchymal cell.

2. on the other hand the mean catalase activity assayed in whole liver homogenate of the same animals equals 279 units per gram of fresh liver tissue. This value is equivalent to 354 units per ml of hepatocyte taking into account the density of the liver as determined by us (d=1,104 g/ml) and a volume ratio of 0.87 between parenchymal cells and total tissue (Greengard et al. 1972).

Consequently the cytoplasmic catalase represents $251 \times 100/354 = 71\%$ of the total catalase activity in the liver parenchymal cell of the guinea pig. Because of the large confidence limits of the microspectrophotometric data, the latter value may vary from 55 to 87%.

Discussion

The linear relationship between enzyme activity and density of DAB reaction product makes this staining technique suitable for the estimation of catalase by microphotometry of semithin sections.

Two important sources of error have emerged during this investigation:

1. Variations in Section Thickness. Their impact is minimized when three sections or more from every specimen equally contribute to the mean.

2. Diffusion Limitation. This phenomenon is well known in immobilized enzyme systems (van Duijn et al. 1967; Thomas and Broun 1973; Barbotin and Thomas 1974). It can be avoided even in model systems containing several times the catalase activity present in guinea pig liver, by a combination of several measures: vigorous shaking, chopper sections not thicker than 40 μm, and increased KCN concentration. When catalase activity is comparatively low, for example in other tissues than liver, or in human liver, diffusion limitation is still less likely to occur.

Another possible error in future investigations may come from the batches of impure diaminobenzidine recently brought on the market by several manufacturers (Sigma, Fluka, Polysciences); this has attracted our attention because we have observed that cryostat sections incubated in this DAB are dirty and cannot be used for direct observation by light microscopy.

The observed reaction kinetics of the staining can be explained assuming that:

1. The enzyme is inactivated in a first order mode during the incubation:

$$dE/dt = -k_1 \cdot E \qquad \text{(I)}$$

and after integration:

$$E = E_0 \cdot \exp - k_1 \cdot t, \qquad \text{(II)}$$

where E_0, E and k_1 represent respectively the initial enzyme activity, the enzyme activity at time t and the first order velocity constant of the inactivation process.

This decrease in enzyme activity could be explained by a) inhibition by the accumulated reaction product. However, this is contradicted by our observations that different activities ultimately lead to quite different amounts of reaction product. b) inactivation of the enzyme by high pH, high H_2O_2 concentration and high temperature. This is confirmed by preincubating liver and model system in medium without DAB at 45° C for 1 h: subsequent incubation in the complete DAB medium did not produce any stain in catalase sites nor of hemoglobin. A similar decline in reaction rate of glutaraldehyde fixed catalase with time in different incubation media has been reported by Lehir et al. (1979). Deactivation of immobilized catalase by H_2O_2 has been described to proceed more quickly at higher temperature (Buchholz and Gödelman 1978; Reuss and Buchholz 1979).

2. The increase in absorbance (dA) per unit time (dt) is linearly related to the enzyme activity (E):

$$dA/dt = k_2 \cdot E = k_2 \cdot E_0 \cdot \exp - k_1 t, \quad \text{(III)}$$

where k_2 represents the zero order velocity constant.

After integration we obtain:

$$A = -(k_2/k_1) \cdot E_0 \cdot (1 - \exp - k_1 \cdot t). \quad \text{(IV)}$$

It can easily be demonstrated from (IV) that after a fixed incubation time t_f the absorbance (A) is linearly related to E_0:

$$A = -(k_2/k_1) \cdot (1 - \exp - k_1 \cdot t_f) \cdot E_0. \quad \text{(V)}$$

For long incubation times ($t \to \infty$) absorbance becomes independent of t:

$$A = -(k_2/k_1) \cdot E_0. \quad \text{(VI)}$$

The model system used in this study closely imitates the cytochemical method as it is applied to tissues, with respect to enzyme source, fixation, incubation conditions, embedding and photometry under oil immersion. Thus the results of the model system can be easily transferred to sections of liver tissue.

From photometry the catalase activity in the cytoplasm of liver parenchymal cells has been calculated in absolute units, and it is in good agreement with the total activity as measured biochemically. For such estimation of catalase only minute amounts of tissues are needed, and the results are not biased by breakage of peroxisomes that occurs during fractionation procedures. Whether particulate (peroxisomal) catalase can be measured photometrically, will depend in the first place on techniques limiting the distributional error caused by the heterogeneity in the measuring field (Ornstein 1952); this problem is instrumental and will not be considered here.

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