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Spectrophotometric Assay of Catalase with Perborate as Substrate[1] (39984)

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In our studies on intracellular localization of enzymes, as determined by density-gradient (including zonal) centrifugation, it is necessary to assay a number of enzymes on as many as 40 samples. For many enzymes, we have used spectrophotometric assays with the spectrophotometer coupled to a key punch (1). For catalase, however, although spectrophotometric assays have been described that use hydrogen peroxide as substrate [for review, see (2)], they are not suitable for assays of numerous tissue samples that by their nature are highly impure; problems such as instability of the substrate, latency of particle-associated catalase, and high blank values made this approach unsatisfactory for our purposes.

Perborate was introduced as a substrate for catalase by Feinstein (3). We used a colorimetric adaptation of this procedure (4) in several studies on catalase distribution (4–6). Solutions of perborate are relatively stable, and the problems of latency are minimal when the assay is carried out at room temperature or higher (7). However, the titrimetric and colorimetric methods are time-consuming, of doubtful accuracy for samples with low activity, and not readily adaptable to our data-processing system.

This report describes the use of perborate as a substrate in a spectrophotometric assay of catalase in mouse liver fractions.

Methods. The reaction mixture, under the conditions finally established, consisted of 2.8 ml of 0.05 *M* potassium phosphate buffer, pH 7.4, and 0.002 to 0.05 ml of a tissue sample diluted to effect an optical density change of 0.10–0.25/min. After equilibration for about 5 min at 30°, the reaction was started by the addition of 0.2 ml of 0.2 *N* sodium perborate, neutralized to pH 7.4 with 85% H_3PO_4 (4). Blanks without perborate were prepared for each sample.

Assays were carried out at 28–30° in quartz cuvettes specially cleaned to minimize the adhesion of bubbles to the surface of the glass (8). Spectrophotometric measurements were made at 220 nm, at intervals of 20–25 sec for a period of 2–3 min, with the apparatus described elsewhere (1).

To test the efficacy of the method, 3 ml of a mouse liver homogenate (1:10 in 0.25 *M* sucrose) was layered over a 42-ml sucrose gradient (0.29–0.88 *M*) containing 0.02 *M* ethanol in a 50-ml polycarbonate centrifuge tube. The tube was centrifuged at 4100 rpm for 70 min; 15 fractions of 3 ml each were collected and assayed. Details of gradient preparation and fraction collection have been previously presented (9). Catalase activity, as indicated by the first-order kinetic constant, was calculated by computing the least-squares fit of a plot of the natural logarithm of the optical density vs time. The slopes of these lines were converted to *k* values in terms of the total sample volume of 3 ml.

Results. Wavelength. Although the maximum absorption of perborate is at 205 nm, the contribution of the buffer and of tissue components is objectionably high at this wavelength. We have found that 220 nm represents the best compromise between sensitivity and blank absorption.

Buffer concentration and pH. Increasing the final concentration of phosphate from 0.047 to 0.093 *M* did not appreciably decrease the rate. However, higher concentrations (0.14 *M* and above) were inhibitory. The pH optimum was 7.40. A 10% decrease in reaction rate was observed when tris-(hydroxymethyl)-methylamine was substituted for phosphate.

Temperature. It has been most convenient to carry out the assays at 30°. At higher temperatures, the initial rates are faster, but as a result of enzyme inactivation the

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reaction obeys first-order kinetics for shorter periods of time.

Miscellaneous properties. Sucrose at final concentrations of 0.1 *M* does not affect the reaction; however, KCl is inhibitory at final concentrations above 0.02 *M*. Triton X-100 cannot be used in this assay because of its high uv absorption. However, digitonin (0.01%) and sodium cholate (0.25%) can be used without interference in tests for activation.

Applications. Figure 1 shows the results obtained with the original homogenate and with various fractions of the gradient. It is apparent that (a) the reaction obeys first-order kinetics over the assay period, (b) in each case the lines obtained by least-squares fit extrapolate to the same intercept, and (c) the slopes of the lines for a given sample are proportional to the volume of the sample used; the actual slopes for H_1 (0.01 ml) and H_2 (0.02 ml) were -6.80 and -13.24×10^{-4} sec^{-1}, respectively.

Table I lists the slopes (with standard errors) and intercepts for assays on the various fractions, calculated on the basis of the total fraction volume of 3 ml. The high values for fractions 1 and 2 reflect the soluble catalase from blood entrapped in the liver. Fraction 1 is the original homogenate layer, and fraction 15 is the pellet. The recovery in fractions 1 through 15 is 108.4% of the original homogenate. The average value of the intercepts is 0.2518 ± 0.0174 (1.286 ± 0.022 optical density units).

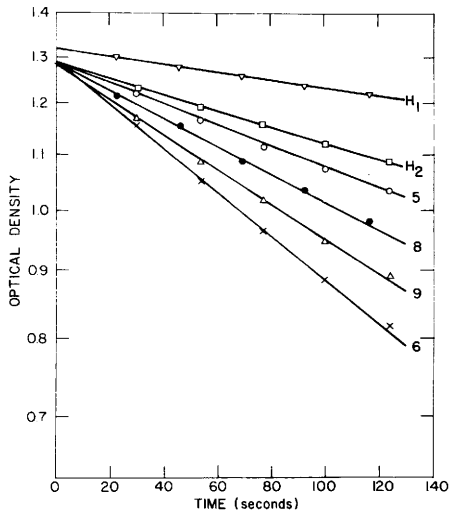

FIG. 1. Catalase assays on a mouse liver homogenate and on four fractions recovered after density-gradient centrifugation. The homogenate was diluted 1:30 in 0.05 *M*, pH 7.0, phosphate buffer; then assayed with 0.01 ml (H_1) and 0.02 ml (H_2) of the dilutions. The fractions were assayed with 0.01 ml (5), 0.02 ml (6 and 8), or 0.04 ml (9).

In a similar experiment, each fraction was assayed for catalase activity by both the spectrophotometric method described above and the colorimetric procedure (4). Regression analysis of the percentage of activity in each fraction as determined by both methods indicated a correlation coefficient of 0.96. The parameters of the distribution curves calculated from the percentages (9) were virtually identical: The midpoint values were 0.487 μm for the spectrophotometric assay and 0.498 μm for the colorimetric, and the corresponding values for the dispersions (σ) were 0.095 and 0.113 μm. These data are evidence that the two procedures are in fact measuring the same phenomenon, disappearance of perborate.

Discussion. The use of perborate as a substrate for the spectrophotometric assay of catalase seems entirely feasible, and superior in many respects to the use of hydrogen peroxide. The principal advantage is the stability of perborate solutions, which obviates the need for repeated standardiza-

TABLE I. PARAMETERS OF CATALASE ASSAYS IN MOUSE LIVER HOMOGENATE AND FRACTIONS.

Fraction	d_m[a]	k ± SE (slope/3 ml)	Intercept
1	–	−0.8586 ± 0.0422	0.2677
2	0.100	−0.5297 ± 0.0202	0.2687
3	0.242	−0.3944 ± 0.0145	0.2760
4	0.316	−0.4460 ± 0.0103	0.2622
5	0.377	−0.5188 ± 0.0133	0.2618
6	0.430	−0.5250 ± 0.0188	0.2582
7	0.478	−0.3857 ± 0.0122	0.2640
8	0.525	−0.3441 ± 0.0054	0.2353
9	0.569	−0.2214 ± 0.0067	0.2592
10	0.612	−0.1298 ± 0.0049	0.2232
11	0.655	−0.0800 ± 0.0033	0.2549
12	0.698	−0.0423 ± 0.0020	0.2206
13	0.741	−0.0364 ± 0.0022	0.2294
14	0.786	−0.0308 ± 0.0011	0.2344
15	–	−2.0053 ± 0.0979	0.2524
Total		−6.5485	
Homogenate		−6.0390 ± 0.0929	0.2611

[a] Mean diameter (micrometers) of particulates in the fraction. See Ref. (9) for calculations.

tion and for correction for spontaneous decomposition.

The spectrophotometric assay is also superior to the titrimetric and colorimetric methods in which measurements are made at a single time point, and in which zero-order kinetics is generally assumed. We are using the method described here routinely in studies on rodent liver peroxisomes.

Summary. A spectrophotometric assay for catalase has been developed in which sodium perborate, rather than hydrogen peroxide, is used as the substrate. The method is convenient, rapid, and readily adapted to the measurement of catalase in subcellular fractions.

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