A SPECTROPHOTOMETRIC METHOD FOR MEASURING THE BREAKDOWN OF HYDROGEN PEROXIDE BY CATALASE*

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Several methods have been developed for following the breakdown of hydrogen peroxide catalyzed by catalase, but these either have not been sufficiently quantitative or have not proved rapid enough to yield reliable data during the critical 1st or 2nd minute of the reaction. Chemical procedures in which residual peroxide is titrated with permanganate (1–3) or an excess of permanganate is measured colorimetrically (4) are accurate except for reaction times of less than a minute, although Lemberg and Foulkes (5) developed a micromethod for obtaining data every 10 seconds (see also Ogura *et al.* (6)). Considerable variability is unavoidable, however, when samples must be taken at such short intervals. The manometric method for measuring oxygen evolved from the system proved in detailed studies to be unsuited for following the rapid breakdown of peroxide in which a diffusion process across the liquid-air interface becomes limiting. This is manifested by changes in both the order of the reaction and the rate of evolution of oxygen with variations in the rate of agitation of the reaction mixture (7). Direct measurement of hydrogen peroxide by polarography provides good quantitative data during the 1st minute of the reaction which fit first order kinetics (8). However, an elaborate, special, electronic circuit is needed for such measurements. Furthermore, as pointed out by Bonnischen, Chance, and Theorell (8), this method appears to give lower values for catalase activity than do titration techniques.

Preliminary experiments for following the breakdown of hydrogen peroxide by observing the decrease in light absorption of peroxide solutions in the ultraviolet were reported by Chance (9) and Chance and Herbert (10). The potentialities of this method have been investigated and a quantitative, spectrophotometric technique for following the breakdown of hydrogen peroxide has been developed for routine studies of catalase kinetics.

EXPERIMENTAL

Spectrophotometric Studies

Since the decrease in ultraviolet absorption by hydrogen peroxide as a function of time is to be used to follow the catalase-peroxide reaction, a

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knowledge of the absorption characteristic of hydrogen peroxide is essential. The absorption spectrum of hydrogen peroxide, measured from 200 to 400 mμ, is shown in Fig. 1. At any wave-length in this range it is possible to use optical density as a measure of peroxide concentration, since the optical density increases linearly with peroxide concentration in accordance with the Beer-Lambert law. The reaction products, oxygen and water, do not absorb light in this spectral region nor does catalase at

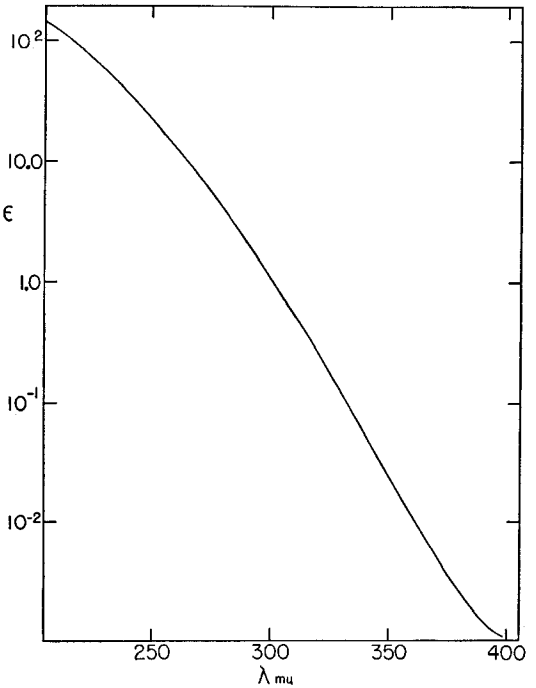

FIG. 1. Ultraviolet absorption curve of hydrogen peroxide in distilled water. ϵ = extinction coefficient = (optical density)/(concentration); optical density = log I_0/I, where I_0 is the incident light and I is the transmitted light intensity.

the concentration employed (usually 10^{-9} M); hence, the ultraviolet absorption is a direct measure of the peroxide concentration in the catalase-peroxide system. If the system contains other substances that absorb in the ultraviolet, the resulting error may be minimized by utilizing a wave-length at which the absorption of the foreign components is a minimum in the region from 200 to 300 mμ, or by preparing a new calibration curve for peroxide to which an appropriate amount of the absorbing foreign substances has been added. An unknown absorbing impurity in the catalase-peroxide system will be revealed not only by an increase in the initial optical density but also by an apparent change in the velocity and order of the reaction.

For computation of velocity constants optical density readings obtained at successive time intervals suffice, and, in a particular experiment, any convenient wave-length can be used, since the velocity constant of a first order reaction is independent of the type of units employed. A convenient initial optical density is between 0.5 and 1.0. In the spectral range from 2100 to 2400 A it is possible to use peroxide concentrations ranging from 5×10^{-4} to 3×10^{-2} M.

Materials and Apparatus

Catalase Preparation—Crystals of beef liver catalase (Worthington) suspended in a saturated ammonium sulfate solution are dissolved by dialyzing against 0.05 M phosphat uffer, pH 7.0. The amber solution is then centrifuged and aliquots of the supernatant are removed for kinetic studies. Provided the buffer is not less than 0.05 M, final concentrations of 10^{-9} M catalase are sufficiently stable at room temperature to permit six to eight successive kinetic trials before appreciable inactivation occurs.

Substrate Preparation—An approximately 5×10^{-3} M solution of hydrogen peroxide is prepared by diluting 0.15 ml. of superoxol (Merck) with 25 ml. of 0.05 M phosphate buffer, pH 7.0.

A Beckman ultraviolet spectrophotometer (model DU) with a temperature-controlled mounting block and three sets of cooling coils, as supplied by the National Technical Laboratories, is used. The mounting block and cooling coil adjacent to the photometer are kept close to room temperature with tap water. The central coils adjacent to the cuvettes are regulated to $\pm 0.1°$ by a thermostatically controlled water bath.

Four quartz 1 cm. cuvettes are used in each set of three kinetic trials. The cuvettes are cleaned with a suspension of magnesium oxide in distilled water by means of a cotton swab, soaked in concentrated nitric acid for 12 hours, then rinsed thoroughly with distilled water, followed by drying with a jet of filtered air or nitrogen. Drying with alcohol is ineffective. Unless adequate precautions are taken to prevent bubbles of oxygen from forming on the cuvette walls during the reaction, serious errors are introduced. Bubbles are especially prone to form if the breakdown of hydrogen peroxide lasts for more than 3 minutes or if any trace of dirt or grease remains on the quartz surfaces of the cuvettes.

Method

Into each of four cuvettes are pipetted 2 ml. of buffered catalase solution; to one, which serves as control, is added 1 ml. of buffer. The slide wire coil is set at the optical density near the initial value for the hydrogen peroxide and the slit width is adjusted to the particular wave-length selected. For routine assays 2400 A has been found to be the most useful wave-length. The selector switch is set at 1.0.

At zero time, 1.0 ml. of buffered hydrogen peroxide is blown from a pipette in a continuous stream directly into the enzyme solution in the cuvette. This insures early and complete mixing of the two solutions in 2 or 3 seconds. The cover is quickly replaced on the cuvette housing unit and optical density readings are taken every 10 seconds (designated

TABLE I

*Comparison of Per Cent Standard Deviations of Chronometric and Tracking Methods for Measuring Catalase Activity**

Method	k_0†	Standard deviation	Per cent deviation
	sec.$^{-1}$		
Chronometric	1.260×10^{-2}	1.05×10^{-4}	±0.84
	1.275×10^{-2}	3.24×10^{-4}	±2.54
	1.098×10^{-2}	1.6×10^{-4}	±1.46
	9.67×10^{-3}	1.25×10^{-4}	±1.29
	1.392×10^{-2}	5.07×10^{-4}	±3.67
	6.90×10^{-3}	2.7×10^{-4}	±3.90
	4.54×10^{-3}	1.36×10^{-4}	±3.00
	4.12×10^{-3}	4.33×10^{-5}	±0.95
Average			±2.2
Tracking	8.77×10^{-3}	2.04×10^{-4}	±2.33
	8.14×10^{-3}	1.32×10^{-4}	±1.63
	8.62×10^{-3}	1.44×10^{-4}	±1.67
	1.325×10^{-2}	1.16×10^{-4}	±0.88
	3.54×10^{-3}	5.0×10^{-5}	±1.40
	1.355×10^{-2}	1.83×10^{-4}	±1.35
	1.355×10^{-2}	2.43×10^{-4}	±1.80
Average			±1.6

* Temperature 20°; pH 7.0; 0.05 M phosphate buffer.

† The velocity constants were determined from an average of six consecutive kinetic trials for each experiment. In three instances one kinetic trial was eliminated from the average because its deviation was more than twice the standard deviation.

the tracking technique). The catalytic process should be followed until at least one-half of the substrate is destroyed in order to facilitate calculating the order and velocity constant. The same procedure is repeated for the other two cuvettes.

Two modifications of the above spectrophotometric technique have been employed which are adapted to special types of catalase studies. In the chronometric modification a first order velocity constant is assumed without consideration of possible changes in the order of the reaction produced by inhibiting processes. The half time of the reaction may be determined

easily with a stop-watch. A specific optical density value is chosen, and, when the galvanometer pointer passes through the null point, the stop-watch is started. The optical density scale is then set at one-half the initial value and the watch stopped when the null point is again passed. The velocity constant is determined from the quotient (ln 2)/(time required to halve the initial density).[1]

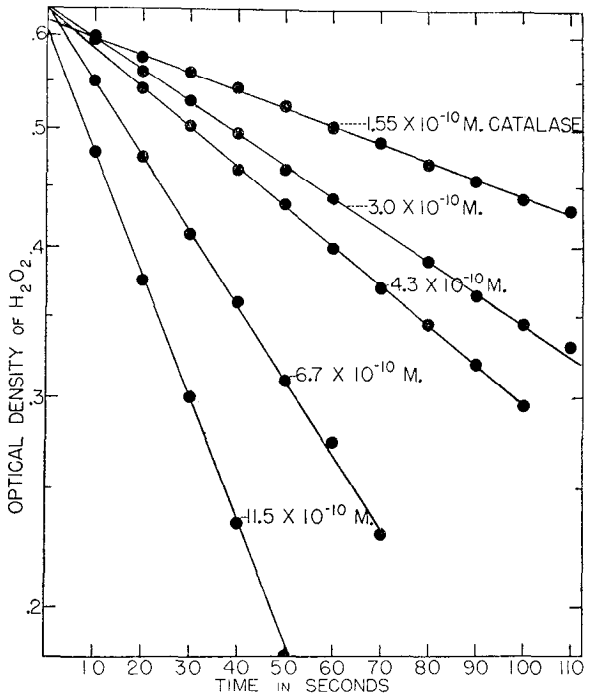

FIG. 2. First order rate curves of destruction of hydrogen peroxide by varying concentrations of catalase (pH 7.0, 0.01 M phosphate buffer, temperature 25.5°, wave-length 240 mμ, initial concentration of hydrogen peroxide approximately 0.015 M).

Table I shows a comparison of the results obtained by the chronometric and tracking methods. The wide variations in standard deviations observed in the former method (0.88 to 3.90 per cent) are attributable to (1) fewer points for determining the velocity constants, (2) deviations from

[1] The second modification utilizes features of both the tracking and chronometric techniques. In view of the uncertainty of the exact zero time of the reaction it is often desirable to select a particular optical density as an arbitrary starting point in order to permit comparison of similar points at a particular time from several curves. This may be accomplished by setting the optical density initially in the same manner as above, but, instead of recording the half time, record the optical densities every 10 seconds.

first order rates caused by inhibitors, and (3) fluctuation in the spectrophotometer. All three sources of error are taken into consideration in the tracking method, thereby permitting calculation of more accurate velocity

TABLE II

Experimental and Theoretical First Order Velocity Constants with Varying Concentrations of Catalase*

Enzyme concentration	k_0, experimental	k_0, theory	Per cent deviation
M	*sec.*$^{-1}$	*sec.*$^{-1}$	
1.55×10^{-10}	3.1×10^{-4}	3.1×10^{-4}	0.00
3.0×10^{-10}	5.9×10^{-4}	6.0×10^{-4}	1.65
4.3×10^{-10}	7.8×10^{-4}	8.6×10^{-4}	9.30
6.7×10^{-10}	1.4×10^{-3}	1.34×10^{-3}	0.45
1.15×10^{-9}	2.3×10^{-3}	2.3×10^{-3}	0.00

* $k_0 = k_s \times E$; $k_s = 2 \times 10^7$ liters mole^{-1} sec.$^{-1}$. E is the molar concentration of enzyme.

TABLE III

Standard Velocity Constant of Beef Liver Catalase

Concentration of enzyme	Observed velocity constant, k_0	Standard velocity constant, k_s
M $\times$ *l.*$^{-1}$	*sec.*$^{-1}$	*l.* $\times$ *mole*$^{-1}$ *sec.*$^{-1}$
24.0×10^{-10}	0.046	1.9×10^7
8.85×10^{-10}	0.0145	1.64×10^7
4.6×10^{-10}	0.0113	2.4×10^7
8.85×10^{-10}	0.0187	2.1×10^7
4.75×10^{-10}	0.0086	1.81×10^7
9.3×10^{-10}	0.0173	1.86×10^7
9.3×10^{-10}	0.0165	1.77×10^7
17.95×10^{-10}	0.035	1.95×10^7
17.95×10^{-10}	0.0324	1.805×10^7
9.3×10^{-10}	0.0198	2.13×10^7
9.3×10^{-10}	0.021	2.26×10^7
9.3×10^{-10}	0.0198	2.13×10^7
9.3×10^{-10}	0.021	2.26×10^7
9.3×10^{-10}	0.0182	1.96×10^7
9.3×10^{-10}	0.0192	2.06×10^7
9.3×10^{-10}	0.0182	1.96×10^7
Average.....		$2.0 \pm 0.17 \times 10^7$

constants. Hence, the average standard deviations are lower (1.6 per cent) and show less fluctuation than in the chronometric method.

For crystalline beef liver catalase the reaction follows first order kinetics over a wide range of enzyme and peroxide concentrations (Fig. 2, Table II) and the reaction remains first order for longer than its half life if the

molarity of the buffer is 0.05 M. The kinetics obtained by the spectrophotometric method are the same as those obtained by other workers (5, 8).

Table III presents values of the first order velocity constant, k_0, obtained by the spectrophotometric method, for a series of concentrations of catalase. The molarity of the catalase was calculated from the extinction coefficient at 4050 A by using the value of 340 $cm.^{-1} \times mM^{-1}$ for horse liver catalase reported by Bonnischen *et al.* (8). The rather wide fluctuations in the catalase activity shown in Table III are attributable to variations in stability of the enzyme in different preparations. The average standard velocity constant, k_s, calculated for 1 M catalase is $2.0 \pm 0.17 \times 10^7$ liters $mole^{-1}$ $sec.^{-1}$ and may be compared with 3.0×10^7 liters $mole^{-1}$ $sec.^{-1}$ reported by Bonnischen *et al.* (8) for horse liver catalase.

Several experiments, performed at different temperatures over the range 11–35°, were used to compute the energy of activation for the catalase-hydrogen peroxide system by means of the Arrhenius equation. The activation energy is quite variable and is sensitive to change in ionic strength and to the presence of small amounts of inhibitors (7, 11). In 0.005 M phosphate buffer, pH 7.0, the experimental activation energy in one series of experiments was 600 calories, a value considerably in excess of the limit of error (±100 calories) of the method employed. This activation energy is distinctly lower than values previously reported (8, 12), and unpublished data indicate that previous investigators may have dealt with partially inhibited catalase which is characterized by much higher apparent activation energies (7). In the case of catalase, however, the measured activation energy does not have the usual meaning, since it is the resultant of the effects of temperature on two different rate-determining processes instead of one.

SUMMARY

A simple, rapid, quantitative spectrophotometric method for following the action of catalase on hydrogen peroxide, based upon the measurement of the ultraviolet absorption of peroxide, is suited to studies of catalase kinetics. By this method the breakdown of hydrogen peroxide was found to follow first order kinetics under a variety of conditions and to increase linearly with catalase concentration. The velocity of this reaction, at low ionic strength, increases only slightly with temperature; the corresponding activation energy is 600 calories.

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