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Catalase Activity

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The destruction of hydrogen peroxide by catalase is probably one of the most intensively studied enzymatic reactions as its abundant literature attests. Those studies, whether based upon titrimetric or manometric procedures have been consistently affected by the fragility of the enzyme; either the reaction-velocity constant decreases throughout the reaction or is constant at only a small fraction of its initial value. Using stronger catalase solutions and modified techniques it has been found that the reaction-velocity constant is large and nearly constant and that some effects previously attributed to the nature of the catalatic reaction were probably due to inactivation of the enzyme. The velocity constant for the reaction of enzyme and substrate has been evaluated in the proper units and the effects of substrate concentration, temperature, pH, and type of catalase upon this velocity constant have been determined. Some of the data obtained previously are re-evaluated and the conclusions are re-interpreted.

The value for the reaction velocity of hydrogen peroxide and catalase during the conversion of hydrogen peroxide into water and oxygen having been re-evaluated, it is compared with the reaction velocity of the formation of the intermediate compound recently found in the catalase hydrogen peroxide reaction [1].

THE MEANING OF KAT. F.

Nearly all of the previous data have been expressed in terms of Kat. F.[2] for this unit is very useful for representing the purity of catalase preparations. Since the data of this paper are in the usual units (seconds and moles per liter) a simple conversion formula has been given below. A similar calculation has

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been made by Haldane [3] and Moelwyn-Hughes [4] but on an iron basis. The factor given by Sizer [5] differs from the one given here.

The kinetics of the disappearance of hydrogen peroxide closely follow the first-order equation, especially when corrections are made for the inactivation of the enzyme [6]. In some manometric experiments an exponential curve is obtained [7, 8], while in others a linear function is found [5, 9, 10]. There are many data which indicate that the zero-order reaction is not due to saturation of catalase with substrate. Also the values of Kat. F. obtained manometrically appear to lie between 0.5 and less than 0.1 of those obtained titrimetrically [11]. In the recent manometric data of George [12], the activity during the initial rapid phase is later calculated in this paper from the values of $\Delta O_2/E$ and from Fig. 2 to be about 2 % of that found here at the same substrate concentrations ($\approx 0.01\ M$).*

Since the highest activity is determined in a first-order reaction, the following equation (1) is probably valid for intact catalase. Further data to support this point will be presented later.

$$\text{Kat. F.} = \frac{\log_{10} \frac{x_0}{x}}{tW/50\text{ ml}} = \frac{k_1}{W/50\text{ ml}} \qquad (1)$$

where x_0 is the substrate concentration at $t = 0$,
x is the substrate concentration at t (minutes),
W is the grams of catalase used in the 50 ml reaction mixture **.

The molecular weight (M) of a number of catalases has been found to be about 225,000 and the proper units, seconds, moles per liter, and natural logarithms can now be used. W grams of catalase per 50 ml is $eM/20$ moles per liter where e is the enzyme concentration. The complete expression for the first-order reaction is:

$$\log_e \frac{x_0}{x} = k_1 e t' \qquad (2)$$

where t' is in seconds. This is only a restatement of Eq. 1 but in proper units. From Eqns. 1 and 2,

$$k' = \frac{\log_{10} \frac{x_0}{x}}{t} = \frac{60\ \log_e \frac{x_0}{x}}{2.3\ t'} = \frac{60\ k_1 e}{2.3} \qquad (3)$$

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* See footnote p. 696.

** The volume is often omitted in this definition; Kat. F. is inversely proportional to the volume employed.

$$\text{Kat. F.} = \frac{520\ k_1}{M} = \frac{k_1}{431} \qquad (4)$$

Eq. 2 is used throughout this paper for evaluating k_1.

Particular forms of Eqns. 3 and 4 have been used by Haldane [3], Moelwyn-Hughes [4], and Sizer [5], to calculate the rate of combination of enzyme and substrate so that this rate might be compared with the theoretical reaction velocity.

Three of the more obvious factors which may cause k_1 to be less than the correct value are:

1. Hematin iron impurities in e.
2. Inactivation of e by x.
3. Partial saturation of e.

At the present time pure crystalline preparations of catalase may be obtained by several methods [13] and accurate spectrophotometric and molecular weight data are available [14, 15]. Factor 1 does not contribute to the error of the present determinations. There is, however, some question as to the number of active hematins in the liver catalases, but in most of these experiments erythrocyte catalase was used. Factors 2 and 3, however, appear to be inadequately studied at present and will be discussed in detail.

ENZYME INACTIVATION DURING THE CATALATIC REACTION

The inactivation of catalase during the decomposition of hydrogen peroxide has been discussed by many workers and many methods have been devised to correct for it by various equations; for a summary see Elliott [16]. It has not been our purpose here to study the inactivation, we have attempted to find the experimental conditions by which inactivation could be minimized. Under such conditions a larger and more accurate value of k_1 may be determined.

Two fairly obvious factors in the inactivation of catalase would seem to be:

1. The spreading of catalase over the various interfaces in monolayers as shown by Harkins, Fourt, and Fourt [17] or by simply increasing the surface area of the reaction vessel by the addition of glass beads (Agner—personal communication).

2. The chemical inactivation of catalase by its substrate by reversible or irreversible reactions.

Both these factors would be more serious in dilute catalase solutions. Thus the amounts of catalase used should be large compared with the amount that will be inactivated at the surfaces of the reaction vessel and its concentra-

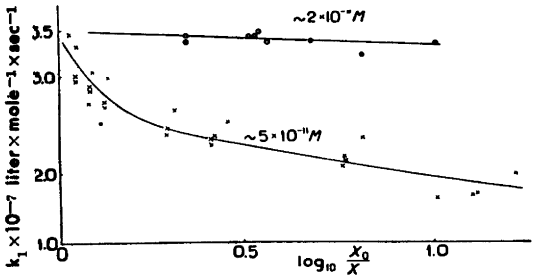

Fig. 1. The effect of enzyme concentration upon the kinetics of the decomposition of hydrogen peroxide by horse blood catalase. The lower curve was obtained using conditions recommended for the determination of Kat. F. (13). 5 mM H_2O_2, 23° C, k_1 calculated by Eq. 2. The values of t may be found from this equation.

tion should be large enough so that each catalase molecule collides with its substrate a comparatively small number of times during the reaction.

Since the half-time required for the decomposition of hydrogen peroxide is inversely proportional to the enzyme concentration, the methods for measuring the activity of stronger catalase solutions must respond rapidly. Only a few of the possible methods are listed below:

1. More rapid manipulation of the titration technique.
2. The use of the flow method
   a. with the platinum microelectrode
   b. with the two-mixer technique.

According to Sumner [13], the enzyme concentration in the determination of Kat. F. is adjusted so that $0.04 > k > 0.025$ where k is in min^{-1} and $\log_{10}$. Using Eqns. 2 and 4 and a value of Kat. F. of 60,000 the enzyme concentration is found to be on the order of $10^{-11}M$ and the half-time of the reaction is about 8 minutes. Typical results for such titrations at 22° C are shown in the lower curve of Fig. 1. At 0° C the inactivation proceeds more slowly [18].

With more rapid manipulation of the titration technique the enzyme concentration can be increased about 100-fold and the half-time decreased to about thirty seconds. The results of the titrations are calculated according to Eq. 2 and are plotted not against time but against the extent of reaction in order that the two curves might be easily compared.

METHOD OF RAPID TITRATION

The same values of k_1 have been obtained over a wide range of conditions and some typical experimental procedures are given here. 2 ml of hydrogen peroxide solution (0.7 ml »Perhydrol» in 50 ml water) are added to 50 ml 0.01 M phosphate buffer (pH = 7) in an Erlenmeyer flask. A 2 ml sample is withdrawn and is blown out into a 2 % sulfuric acid solution and titrated with 0.01 N permanganate to give the initial peroxide concentration.

The catalase solution is diluted in 0.01 M phosphate buffer (pH = 7) to about 1 μM and its density is measured at the peak of the Soret band in the Beckman spectrophoto-

meter. About 0.03 ml of this solution is pipetted into a watch-glass. Simultaneously the stop-watch is started, the watchglass is dropped into the flask containing the substrate, and the liquid in the flask is rapidly swirled.

At 13 seconds a 2 ml sample is withdrawn and is rapidly blown into a swirling 2 % sulfuric acid solution. The time at which the pipette is blown out is noted. When clean pipettes are used the differential error in delivery from the pipettes is less than 11 %. A second sample is withdrawn at 28 s and a third at 43 s. The values of k_1 are calculated by Eq. 2 and may or may not be extrapolated to $t = 0$.

A satisfactory alternative procedure is to withdraw the first sample a few seconds after adding the enzyme and then to take a second sample fifteen seconds later. k_1 is calculated using the ratio of the two values of hydrogen peroxide concentration and the time interval between the measurements. Although k_1 is assumed to be a first-order constant over this interval, this method has the advantage that the slowness of mixing enzyme and substrate causes no error and the error in timing is only the differential error of the two measurements.

In other cases the reaction has been stopped by blowing a few ml of sulfuric acid into the reaction vessel at the desired time. No appreciable difference in the values of k_1 was found with these variations of the method.

Fig. 1 shows a striking difference between the course of the two reactions; with dilute enzyme the initial portions of the curve are sharply inflected and catalase inactivation occurs which is soon complete. The residual activity is relatively constant and is the phase of the catalatic reaction upon which some of the catalase activity data has previously been based. It seems, however, that this residual activity might not have the same characteristics as intact catalase and therefore the curve found with the stronger enzyme is preferable. In this case there appears no abrupt decrease in activity up to the earliest time measured. The slight inactivation that occurs during the course of the reaction is at a constant rate and no difficulty is encountered in extrapolating to an initial rate. In fact, there is so little difference between the rate obtained in the early portion of the reaction (between 15 and 30 s) and that obtained by extrapolation (3 %) that the unextrapolated value may be used in nearly all cases.

It appears that the curve with dilute enzyme could possibly be extrapolated to the same initial activity as obtained with the stronger enzyme. The spread of the experimental data attest the inaccuracy of such a procedure. On the other hand, extrapolation from points obtained at 3, 6, and 9 minutes as recommended by Sumner [13] gives a definitely smaller activity (about three quarters).

The data of Fig. 1 were not taken with sufficient time resolution to prove that the stronger catalase solution did not have a similar initial decrease of activity in the first few seconds of the reaction. The activity of strong catalase solutions was therefore measured by a quite different technique. A modified

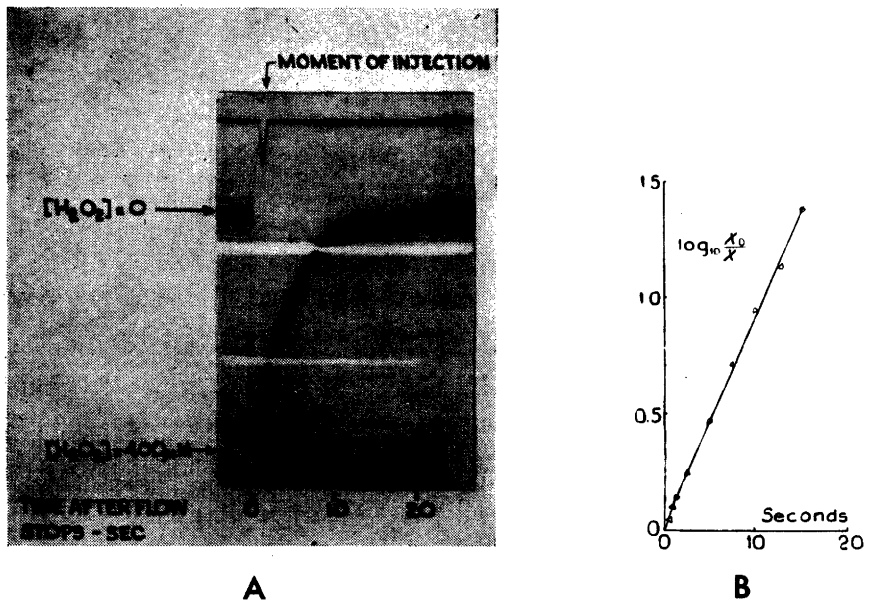

Fig. 2. The kinetics of the catalase reaction measured by the platinum microelectrode with anodic polarization. In A is shown the original data recorded by a mirror oscillograph and in B the graph of the data according to the first-order equation. 5.7 × 10⁻⁹ M horse liver catalase, 400 μM hydrogen peroxide, 0.01M phosphate buffer, pH = 6.5, T = 25° C, electrode polarized at +1V, $k_1 = 4 \times 10^7$ liter × mole^{-1} × second^{-1}. Expt. 90 C.

circuit for the platinum microelectrode [19] gives a linear response to the hydrogen peroxide concentration within a fraction of a second. A very small electrode has been inserted into the capillary cuvette of the rapid flow apparatus so that advantage may be taken of the rapid mixing. A typical record and a logarithmic plot of the data are shown in Fig. 2A and B. The moment of injection of the mixed enzyme and substrate solutions into the capillary is shown on the top trace of Fig. 2A. The electrode current suddenly increases due to the initial hydrogen peroxide concentration as shown by the sharp drop in the lower trace. The flow stops in about one second and the catalatic reaction proceeds along an exponential curve. As the logarithmic plot of Fig. 2B shows, there is no abrupt discontinuity which would indicate that catalase has a higher activity in the first few seconds of the reaction. Thus the data of Fig. 2B fully justify the straight-line extrapolation used in the upper curve of Fig. 1. Also the value of k_1 for Fig. 2B is 4×10^7 liter × mole^{-1} × second^{-1} in fairly good agreement with the data of Fig. 1. The results with

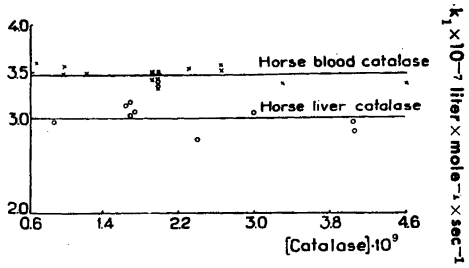

Fig. 3. The effect of enzyme concentration upon k_1 for liver and blood catalases. 5 mM H_2O_2 20° C. t = 15 sec.

the platinum electrode are somewhat less accurate than those obtained by titration and a typical series of data is shown later in Table 7. No significance is yet attached to the small numerical differences between the values of k_1 determined by the two methods.

It is of importance to learn just how much enzyme is required to give negligible inactivation in the first fifteen seconds of the reaction. This is illustrated by Fig. 3 which shows a substantially constant value of k_1 for the range of enzyme concentration tested. With more dilute enzyme the value of k_1 decreases as might be expected from Fig. 1. The value of k_1 was constant from 10^{-9} to 10^{-8} M catalase when using the platinum electrode technique in the capillary of the flow apparatus.

It is clear that only a portion of catalase is intact when its activity is measured by the older methods and that many of the factors previously found to affect the activity of catalase may only have affected the rapidity of its inactivation. One would first expect this factor to be clearly demonstrated in experiments in which the substrate concentration is varied for there the number of times that each catalase molecule must react will vary greatly.

THE EFFECT OF SUBSTRATE CONCENTRATION UPON THE ACTIVITY OF CATALASE

The substrate-activity characteristic of an enzyme is of great fundamental importance since it gives much information on the enzyme mechanism. If the reaction velocity is governed by the rate of breakdown of an enzyme-substrate compound in accordance with the Michaelis theory, then the Michaelis constant for such a complex must be determined in order to calculate the absolute reaction velocity (see Factor 3). From this point on the distinction between a hypothetical Michaelis compound governing the rate of destruction of hydrogen peroxide and the recently reported catalase hydrogen peroxide complex [1] must be kept clearly in mind. The latter compound is present at its saturation value during the destruction of hydrogen peroxide but the rate

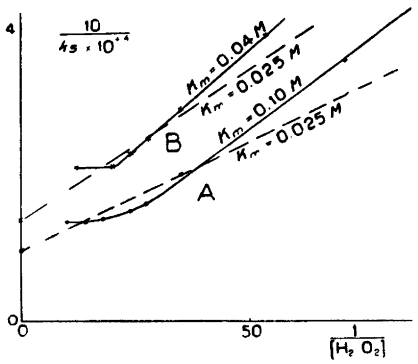

Fig. 4. The data of Euler's (20) Table 5 (circles) and Table 7 (crosses) plotted in accordance with Lineweaver and Burke's (22) equation, $\frac{1}{V} = \frac{1}{x_0} \cdot \frac{K_m}{V_{max}} + \frac{1}{V_{max}}$ *where* $\frac{10}{k \cdot s \cdot 10^4} = \frac{1}{V}$. *The dashed lines are drawn in accordance with the values of* $\frac{1}{V_{max}}$ *and* K_m *shown on Figures 2 and 3 of (20).*

of its monomolecular breakdown does not govern the catalatic activity. To avoid confusion this catalase-hydrogen peroxide complex will be consistently termed »intermediate compound» while the hypothetical Michaelis compound will be termed »Michaelis compound».

Although the mere existence of a logarithmic relation between substrate concentration and time rules out the possibility of significant amounts of a Michaelis compound in catalase action, many investigators have neglected this fact and have attached much significance to the maximum found in the activity-substrate relation; it has been concluded that the activity of catalase involves a Michaelis compound of $K_m = 0.025$ M[20] and 0.033 M[21]. It appears to be desirable to review the data on which those conclusions were based.

Following the experience of Lineweaver and Burke [22] that Eq. 5 gives the better placement of experimental data at low substrate concentrations,

$$\frac{1}{V} = \frac{1}{x_0} \cdot \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \qquad (5)$$

the data of v. Euler [20], Stern [21], and Williams [6] have been replotted in Figs. 4, 5, and 6. Lineweaver and Burke [22] have previously made a plot of Stern's data

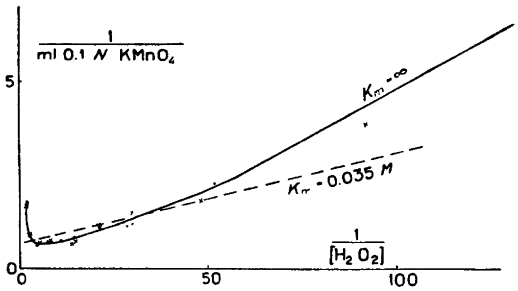

Fig. 5. The data of Stern's (21) Figures 1 and 2 (points) and 3 and 4 (crosses) plotted as in Fig. 4 except $\frac{1}{ml\ 0.1\ N\ KMnO_4} = \frac{1}{V}$ *and the values of S are converted into moles per liter. The dashed line corresponds with the value of* K_m *given by Stern (0.033 M). The data of Figures 2 and 4 are adjusted to the scale of Figures 1 and 3 respectively by means of the values of* V_{max} *shown on Stern's figures.*

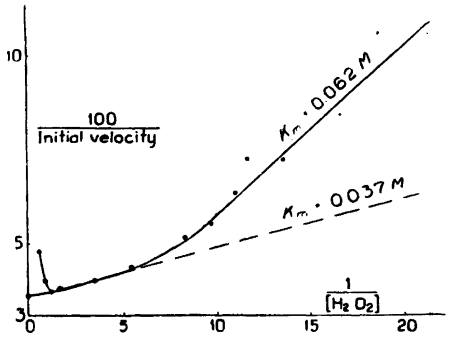

Fig. 6. The data of William's (6) Figures 3 and 3a plotted as in Figure 4 except $\frac{100}{M/L\ H_2O_2/min} = \frac{1}{V}$. *The dashed line gives the value of* K_m *that might have been determined by Williams had he reasoned as had Euler and Stern.*

using a form less suited to low substrate concentrations (Fig. 3 B II′ [22]) and, by neglecting those data at lower substrate concentrations, have obtained values of K_m and V_{max} which are later used to show that an active and an inactive intermediate compound may satisfy Stern's catalase data. We prefer to emphasize the data at low substrate concentrations and to evaluate the slopes of Figs. 4, 5, and 6 for large values of $\frac{1}{[H_2O_2]}$ which give values of K_m of 0.07, ∞, and 0.62 *M*.

These values indicate that the Michaelis constant for catalase activity, if it exists, is considerably greater than previous authors had assumed.

Fig. 7 affords an explanation of these effects. Here the reaction velocity obtained in the 15 s titration of strong enzyme (A) is compared with that obtained in the 3, 6, and 9 minute titration of dilute enzyme (B) at various values of substrate concentration. Curve A clearly corresponds to $K_m > 1\ M$ in agreement with the large values obtained above. The severe inactivation of the enzyme is clearly indicated by the difference between curves A and B. While the bulk of Stern's data was consistent with Lineweaver and Burke's mechanism, their proposal is quite inadequate to explain the effect of varying enzyme concentration demonstrated by curves A and B. There appears as yet to be little evidence supporting the existence of significant concentrations

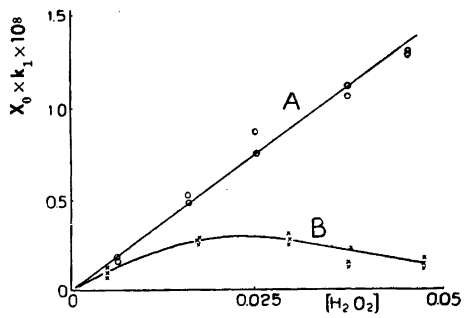

Fig. 7. The variation of reaction velocity with substrate concentration. The circles are obtained with rapid titration (15 sec) in strong enzyme while the crosses were obtained according to the previous methods with dilute enzyme and measurements at 3, 6, and 9 minutes (from top to bottom). Horse blood catalase, 3° C, pH 6.8, 0.01 M phosphate.

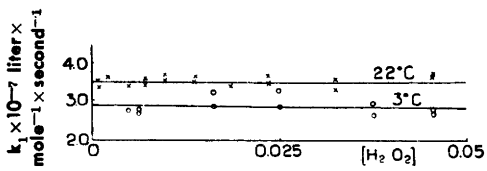

Fig. 8. The effect of a variation of initial substrate concentration upon k_1, at two temperatures as determined by 15 sec. titrations. Horse blood catalase pH = 6.8, 0.01 M phosphate.

of active or inactive compounds of the type postulated by Michaelis or by Lineweaver and Burke. On the other hand the peroxidatic catalase — hydrogen peroxide intermediate compound is readily demonstrable [1], but is saturated with substrate over the entire range that the activity has been studied and therefore does not cause the reaction velocity to be affected by substrate concentration.

Since the constancy of k_1 and not its variation with substrate concentration is to be evaluated, the data of Fig. 7 are replotted and repeated at room temperature as shown in Fig. 8. The constancy of k_1 is apparent. It is of considerable interest that the temperature coefficient of k_1 is independent of substrate concentration since this affords additional proof of the absence of an appreciable concentration of a Michaelis compound.

Similar results are readily obtained with the platinum microelectrode and are shown in Fig. 9. Not only is k_1 independent of substrate concentration but its numerical value is approximately the same as in Fig. 8. At lower substrate concentrations there is an apparent increase of k_1 which is believed to be due to errors in the electrode.

A number of years ago the activity of strong catalase was studied by a two-mixer technique similar to that used by Hartridge and Roughton [23] in hemoglobin studies*. Since the results can now be readily interpreted, they are included here. Buffered enzyme and substrate were mixed and caused to flow through a tube at constant velocity [24]. After a distance of flow equivalent to 0.2 s the catalatic reaction was stopped in a second mixing chamber

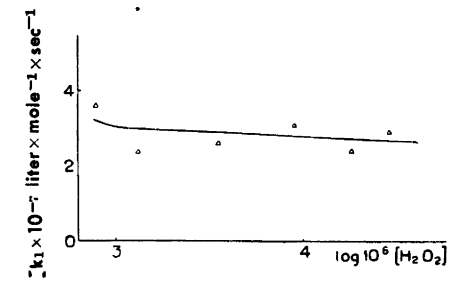

Fig. 9. The effect of a variation of initial substrate concentration upon k_1 as determined by the platinum microelectrode 5.9 × 10^{-9} M/L horse liver catalase, Expt. 87a, pH = 6.5, 0.01 M phosphate, 25° C.

* These experiments were mainly the work of the late G. A. Millikan who was assisted by P. McLaughlin and were carried out at the Johnson Foundation for Medical Physics, University of Pennsylvania.

by the addition of sulphuric acid. A sample of the reaction product was then titrated with permanganate.

After some difficulty due to the decomposition of hydrogen-peroxide in the storage syringe was eliminated by coating the parts with glyptal, the data of Table 1 were obtained.

Table 1. Activity determinations by the flow method.

x_0, moles per liter	0.05	0.3	1.0
x_0/x	1.25	1.17	1.22
t—s	0.2	0.2	0.2
k—s^{-1} ($\log_e$)	1.2	0.78	1.1
Catalase dilution	100	100	100
$k \times$ dilution	120	78	110

From the values of k the catalase concentration was about 3×10^{-8} M. The value of $k \times$ dilution obtained by the usual 5-minute titration was only 65. These tests therefore confirm these later data and show that the reaction velocity constant of very strong catalase is nearly independent of the substrate concentration up to 1 M and is in excess of that obtained by the old titration method.

These three independent methods show no variation of k_1 in excess of the experimental error over the entire range of substrate concentration tested. The properties of catalase found by these methods differ considerably from those obtained with more dilute enzyme and this difference may be due to enzyme inactivation or to the properties of partially-inactivated catalase. In any case these data give no support for the existence of an appreciable concentration of a Michaelis compound (see bottom of p. 691).

The recent note of George [12] on manometric studies of catalase activity adds support to these data in the region where a comparison is possible. Apparently the rate of initial oxygen evolution R_r, which precedes the enzyme inhibition, is linearly related to the initial substrate concentration up to 0.3 M hydrogen peroxide as shown by the data of Table 2. Georges' data, which clearly do not fit a hyperbolic relationship required by the Michaelis theory, do agree with the experiments of this paper.

Table 2. The variation of the initial rate of oxygen production in George's [12] manometric experiments (Fig. 2).

Initial hydrogen peroxide (x_0—M)	0.05	0.1	0.15	0.2	0.3
R_r as measured from Fig. 2 (μl/min)	290	640	1100	1400	2100
$R_r/x_0 \times 10^{-3}$ (μl $\times$ min^{-1} $\times$ liter $\times$ $mole^{-1}$)	5.8	6.4	7.3	7.0	7.0

Above this concentration an inhibition is observed that was not found in the titrimetric experiments of Table I and resembles the older titration data with dilute enzyme.

It may be that the conditions of the manometric experiment, rapid shaking over a large surface, are more severe than in these experiments and that some inhibition of catalase activity occurs even during the initial phase of oxygen evolution. A calculation of k_1 is desirable.

Although the enzyme concentration used in George's experiments is not given, it may be estimated from the value of $\Delta O_2/E \approx 1 \times 10^6$ moles oxygen per mole catalase and the value of $\Delta O_2 = 460$ μl in 0.5 M hydrogen peroxide (see Fig. 1A). $E \approx 2 \times 10^{-5}$ μ moles*.

The quantity R_r/x_0 of Table 2 is converted into these units: $\frac{7.0 \times 10^3}{22.4 \times 60} =$ 5.2 μmoles $\times$ second^{-1} $\times$ liter $\times$ mole^{-1}. The absolute reaction-velocity constant, k_1, is equal to $\frac{dx}{dt} \cdot \frac{1}{x_0\, e}$ in the initial phases of the reaction. Since $\frac{dx}{dt} =$ R_r and x and e are both in μmoles, $k_1 \approx 2 \times 10^5$ liter $\times$ mole^{-1} $\times$ second^{-1} which is roughly 1 % of the value found in these tests (3×10^7 liter $\times$ mole^{-1} $\times$ second^{-1}.

The value of k_1 for the steady rate at 0.05 M hydrogen peroxide is considerably smaller, $\approx 5 \times 10^4$ liter $\times$ mole^{-1} $\times$ second,$^{-1}$ corresponding to 0.2 % of the full activity of catalase.

In accepting the data of Table 2 in support of these experiments it must be assumed that the small fraction of the initial catalase concentration active in George's experiments had the same characteristics as the intact catalase of these experiments.

THE EFFECT OF TEMPERATURE UPON THE VELOCITY OF THE CATALATIC REACTION

Another important characteristic of the catalase reaction on which misleading data may have been obtained is the temperature coefficient.

The importance of the order of the overall enzymatic reaction upon the interpretation of thermal data has been emphasized by Sizer [25] since the tem-

* A letter from Dr. George indicates that the actual enzyme concentration was 7.4×10^{-6} μ moles in a volume of 4 ml. Thus $\frac{\Delta O_2}{E}$ was near its minimum value (0.3×10^6) in his Fig. 1 A and k_1 is 7×10^5 liter $\times$ mole^{-1} $\times$ second^{-1} about 2.5 % of this value. Dr. George prefers to explain this discrepancy by stating that his enzyme preparation had a smaller activity than that of these crystalline preparations.

perature coefficient of a zero-order overall reaction must represent the temperature coefficient of the break-down of an intermediate compound while the temperature coefficient of a first-order overall reaction usually represents the temperature coefficient of the combination of enzyme and substrate. In Sizer's recent measurements of the temperature coefficient of the catalase reaction [5] the manometric data closely approximate a zero order reaction. In other manometric experiments the values of Kat. F. have been found to be low and variable [11, 12].

Using the titration technique discussed previously a series of values for the energy of activation (μ) have been obtained as shown by Table 3. It would be desirable to prove the constancy of μ over this temperature range but the small change of velocity taxes the accuracy of the titration method.

Table 3. Titration data on the energy of activation of the catalase-hydrogen peroxide reaction.

Type of Catalase	T_2 (°C)	T_1 (°C)	$(k_1)_2 \times 10^{-7}$	$(k_1)_1 \times 10^{-7}$	μ cal
Horse blood	25.5	1.5	3.50	2.66	1880
Horse blood	23.5	7.2	3.50	3.11	1490
Horse blood	22.0	2.0	3.54	2.84	1800
Horse blood	23.5	3.0	3.50	2.86	1760*
Horse liver	22	2.0	3.00	2.55	1320

The values are considerably smaller than those obtained by Sizer (4200 cal)[5]. The values obtained by Williams [6] are closer to the present values than those of other workers. Apparently the energy of activation for the reaction of intact catalase with its substrate is less than that for the reaction of partially inactivated catalase studied by previous workers.

It is of interest to recalculate the theoretical value of reaction velocity of catalase and hydrogen peroxide on the basis of the value of μ for horse blood catalase. This calculation closely follows the pattern of Sizer [5] with two important exceptions; $\mu = 1700 \pm 100$ calories, $k_1 = 3.5 \times 10^7$ liter $\times$ mole^{-1} $\times$ second^{-1}.

The number of activated collisions of enzyme and substrate per second (or the number of substrate molecules destroyed per second) is

$$k = \frac{3}{13} \bar{\mu}\, n_e n_x \pi \sigma^2 \Sigma^{-\mu/RT} \qquad (5)$$

when n_e and n_x are the numbers of enzyme and substrate molecules per ml. Since k is calculated from the activity data as follows,

* See Fig. 8.

$$k = n_x \frac{\log_e \frac{x_0}{x}}{t} = n_x k_1' e = n_x k_1' \frac{10^3 n_e}{6.06 \times 10^{23}} \qquad (6)$$

Eq. 6 may be simplified by eliminating k and solving for k_1',

$$k_1' = 1.4 \times 10^{20} \bar{u}\, \pi\sigma^2 \Sigma^{-\mu/RT} \qquad (7)$$

Thus the teoretical reaction velocity constant, k_1' (liter $\times$ mole^{-1} $\times$ second^{-1}), for the rate of reaction of enzyme and substrate is seen to depend only upon the velocity of the substrate molecule $\bar{u}$ (assuming a stationary catalase molecule), the area of the catalase molecule (here taken to be the entire area $= \frac{u\sigma^2}{4}$) and the fraction of collisions that are effective ($\Sigma^{-\mu/RT}$). Using the values of $\bar{u}$ and σ calculated by Sizer [5],

$$k_1' = 1.24 \times 10^{13} \Sigma^{-\mu/RT} \qquad (8)$$

For $\mu = 1700$ and $T = 300°$ K, $\Sigma^{-\mu/RT}$ is 1/17 and

$$k_1' = 7.3 \times 10^{11} \text{ liter} \times \text{mole}^{-1} \times \text{second}^{-1} \qquad (9)$$

If this value is decreased by 5.2×10^{-3} on the basis that the active center has the area of four iron atoms as suggested by Sizer [5], a value of 4×10^9 is obtained which is 100 times larger than the observed rate. The accuracy of the theory underlying the theoretical calculation is probably not high, especially since Moelwyn-Hughes [26] mentions in a calculation of the molecular statistics of saccharase action that the theory of Hinchelwood would possibly increase the collision number by several powers of ten.

Reasonable conclusions of this comparison are: — 1. the experimental value for the reaction of catalase and hydrogen peroxide (3.5×10^7 liter $\times$ mole^{-1} $\times$ second^{-1}) is considerably less than the smallest theoretical value that can be calculated on the basis of reasonable assumptions — and 2. the probability of an effective collision of enzyme and substrate is extremely large ($\Sigma^{-\mu/RT} = 6$ %).

It appears that an even larger fraction of the collisions of oxygen and hemoglobin are effective for in this case Hartridge and Roughton [23] found the reaction-velocity change to be within their experimental error over the 10° interval. The latter was calculated to be 2.4 % from the nine data of their Table 2 (2900 ± 70, probable error of arithmetic mean). The value of μ must therefore be less than 350 cal and more than 50 % of the collisions are effective.

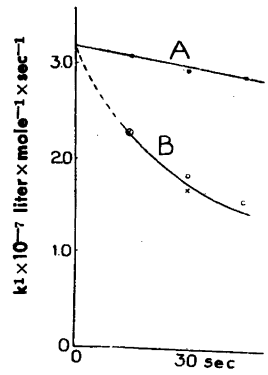

Fig. 10. The effect of pH upon the reaction velocity constant (k_1). Curve A, pH = 7.0; Curve B, pH = 3.5 0.001 M phosphate (acidified with phosphoric acid).

A similar situation exists in the reaction of peroxidase and hydrogen peroxide when the following preliminary data were obtained [27], but were previously unpublished since the error was relatively large.

Table 4. Preliminary values of the effect of temperature upon the formation of the peroxidase-hydrogen peroxide complex [27].

T°C	3	25	42
$k_1 \times 10^{-6}$ liter × mole^{-1} × second^{-1}	8	9	9

There is no doubt that an appreciable percentage of the collisions is effective.

These data therefore indicate that a large fraction of the collisions of enzyme and substrate are effective.

THE EFFECT OF pH

When a neutral catalase solution is diluted in a neutral phosphate buffer the turnover of substrate proceeds with very little decrease of k_1 as upper Curve A of Fig. 10 shows. If an acid phosphate solution is used, k_1 falls from its initial value at pH = 7 to much lower values as Curve B of Fig. 10 shows. Apparently there is no immediate inhibition of catalase by the acid solution; but there is a progressive decrease of the enzyme activity during the course of the reaction as if the inactivation of catalase by hydrogen peroxide were proceeding more rapidly in acid solutions.

The data of Table 5 show that a portion of the activity is lost by diluted catalase in the absence of substrate but that a much more extensive loss occurs when substrate is present. The inactivation of catalase in the presence of substrate can also be greatly increased by acetate in neutral solution as the

data of Table 5 show. Phosphate and formate do not cause much loss of activity in neutral solutions. As a check of these data the equilibrium constant for formate inhibition is calculated to be 3.7×10^{-3} *M* (pH = 7, 7° C) and is compared with 4.2×10^{-3} *M* obtained by extrapolating and interpolating the data of Agner and Theorell [28] for pH and temperature respectively.

Table 5. The effects of dilution at pH = 7 and 3.5 upon the activity of catalase. Horse blood catalase, $k_1 = 2.85 \times 10^7$ liter $\times$ mole$^{-1} \times s^{-1}$ at 7° C. 5 mM hydrogen peroxide, 0.001 M phosphate buffer, acidified with phosphoric acid to give pH = 3.5.

Treatment of catalase	pH	$k_1 \times 10^7$ (by 30 s titration)	% Activity remaining
Diluted to 10^{-9} for 9 min and then hydrogen peroxide was added for the activity test.	7.0	2.32	79
Ditto	3.5	0.94	32
Diluted to 10^{-9} in 5 *mM* hydrogen peroxide for 9 min and then fresh hydrogen peroxide was added for the activity test.	7.0	1.96	66
Ditto	3.5	0.40	14

Table 6. The effect of various anions upon the decrease of k_1 during the decomposition of 5 mM hydrogen peroxide at pH = 7.0. In both cases the activity was tested after 9 min. by the addition of fresh substrate (30 sec. data).

Conditions of experiment	0.01 *M* phosphate	0.16 *M* formate	0.37 *M* acetate
Percent activity remaining after 9 min. dilution to 10^{-9} *M* in solution containing anion alone.	79	88	75
Percent activity remaining after 9 min. dilution to 10^{-9} *M* in solution containing both anion and 5 *mM* initial hydrogen peroxide.	66	81	12

THE RELATION BETWEEN THE NUMBER OF INTACT HEMATINS AND ACTIVITY

The fourth important characteristic of catalase activity is the number of hematins that are required for maximal activity. This question has been studied by Lemberg [29]. Although their values of Kat. F. were carefully cor

rected for enzyme inactivation, they are considerably less than values obtained by these methods and therefore some of the inactivation may not have been accounted for.

Since pure horse blood and liver catalases are now available and have been proved to have identical protein components [15], the comparison has been made on the basis of these two preparations instead of upon liver catalases of varying bile pigment content [24]. The titration data of Fig. 3 show that the ratio of the activities of horse-liver to horse-blood catalase is 0.86. The platinum electrode data of Table 7 give an average ratio of 0.89. These enzyme concentrations were determined on a dry weight and molecular weight basis which was in agreement with the spectrophotometric determination of concentration at the peak of the Soret band.

These activity ratios are much larger than those computed by Lemberg [29] and suggest that these liver catalases contain considerably less bile pigment. The spectra of the carbon monoxide hemochromogens obtained from these catalases have been compared with that of Lemberg's [29] Fig. 2. The ratio of bile pigment to total heme for this horse-liver catalase is calculated by Lemberg's formula [29] $[(\Sigma_{630}/\Sigma_{570}) - 0.047]/[(0.55\Sigma_{630}/\Sigma_{570}) + 0.96]$ where the second term of the numerator has been increased to equal $\Sigma_{630}/\Sigma_{570}$ for horse blood catalase carbon-monoxide hemochromogen. A value of 9 % is found which is equivalent to 91 % non-bile-pigment hemoprotein.

The hemochromogen spectra of these catalases also differ markedly from that found by Lemberg, the value of $\Sigma_{650}/\Sigma_{557}$ was 0.25 in his experiments while this horse-liver catalase preparation gives a value of only 0.11. By correcting both ratios for that of horse-blood catalase (0.035) and by assuming that Lemberg used a catalase of the same bile pigment content for this test as for Fig 2 [29] a ratio of bile pigment to total heme of 14 % is calculated for this horse-liver catalase or 86 % non-bile-pigment hemoprotein. These two values appear to be in good agreement with the ratio of the hematin iron content of horse-liver and blood catalases as determined by pyridine hemochromogen (83 %, Bonnichsen [15]).

Although these ratios are in agreement with Agner's early determinations of the protohematin content of liver catalase [30], his later values [14] and Theorell and Agner's [11] magnetic titration data gave values of about 75 %. Agner and Theorell [28] found the ratio of the extinctions of liver and blood catalase fluorides to be 81 % (calculated from their Fig. 4) until m/40 fluoride concentration was reached when a value of about 75 % was obtained. A similar spectrophotometric test with cyanide gave 75 %.

DISCUSSION

A fifty-fold increase in the amount of catalase used in activity determinations appears to have made inactivation and other effects unimportant. The values of activity can be determined without introducing a personal element into the determination by extrapolating a sharply inflected curve. Since the values of activity determined by this method are quite independent of variations of initial substrate concentration, are only slightly influenced by temperature changes, and give a constant activity for a wide range of enzyme concentrations, it is recommended that future determinations of catalase activity be made in this way. No set procedure has been formulated simply because the range of conditions over which constant activity may be obtained is relatively large. The value of k_1 is constant in the titrimetric method from 0.6 to 5 $\times$ 10^{-9} M catalase. In the platinum electrode tests which were carried out in the 1 mm capillary of the flow-apparatus k_1 was constant from 10^{-9} to 10^{-8} M catalase*.

Since the catalase concentration is now readily calculated on a molar basis either from the known molecular weight or from spectrophotometric data, it is suggested that the results obtained by this new method be given in terms of k_1 (see Eq. 2), the velocity constant for the disappearence of hydrogen peroxide, instead of Kat. F. There are two reasons for this:

1. Values of Kat. F. determined in this manner are consistently higher unless the inflected curves obtained by the old method are optimistically extrapolated. In order to avoid unnecessary qualifications as to how the activity was determined, a change of units is desirable.

2. The quantity k_1 is expressed in accepted units and has a definite physical-chemical meaning; the activity of catalases expressed in this manner are immediately comparable with data on other enzymes.

The value of k_1 is found to be 3.5 $\times$ 10^7 liter $\times$ mole^{-1} $\times$ second^{-1} at 22° C for horse blood catalase and corresponds to a Kat. F. of 61,000 at 0° C. From Fig. 1 k_1 found by extrapolation of the 3, 6, and 9 minute data is only 2.75 $\times$ 10^7 liter $\times$ mole^{-1} $\times$ second^{-1} or a Kat. F. of 48,000 at 0° C. However, the Kat. F. actually found at 0° C for this catalase by the 3, 6, and 9 minute extrapolation [15] was 65,000. This discrepancy may be caused by an extrapolation error or by a change in the shape of the catalase kinetics from the sharply inflected curve of Fig. 1 to a more slowly changing curve at 0° C (see, for example, Sumner [18]). Extrapolation errors and the change of the rate of inactivation of catalase with temperature, initial substrate concentration, pH, etc.,

* It is surprising that this is so for the ratio of area to volume in the capillary is probably an order of magnitude greater in the capillary than in the flasks used for the titration experiments. Air-water interfaces are, however, absent.

make the older method of activity determination very dangerous to use for a comparison of catalase activities or for kinetic studies. It is therefore recommended that the 3, 6, and 9 minute extrapolation be abandoned in favor of this titration method and that the activity values be given in terms of k_1 at 20° C for enzymes of known molecular weight (horse liver [31] and beef liver [32], horse erythrocyte [15]). Since the correct values of k_1 are given in this paper for horse liver and blood catalases, the enzyme concentration (e) can be readily determined by an activity test and is calculated from Eq. 2. The enzyme concentration of a large number of purified preparations have been determined in this manner and have been found to be equal to that determined in the Beckman spectrophotometer at the peak of the Soret band using $\Sigma = 380$ $cm^{-1} \times mM^{-1}$ for horse-erythrocyte catalase and $\Sigma = 340$ $cm^{-1} \times mM^{-1}$ for horse-liver catalase prepared according to Bonnichsen [15]. The purity of the preparation is then expressed as the ratio of e found by activity test or spectrophotometrically to the enzyme concentration found from the dryweight and molecular weight. The spectrophotometric method of determining the enzyme concentration is more rapid than the activity test and is, therefore, often used as a test of the purity of the preparation. The final stage of purification is, however, best judged by the extinction coefficient at 280 mμ and has been found to be $\Sigma = 280$ $cm^{-1} \times mM^{-1}$ for the horse blood and liver preparations [15].

For catalases of unknown molecular weight Kat. F. is still useful but this titration procedure should be used.

This new method for obtaining catalase activity now gives data on the enzyme mechanism which do not appear to be obscured by inactivation effects. Therefore four of the fundamental factors in the mechanism of the enzyme have been re-examined and new conclusions have been reached in three cases. It appears that several other conclusions on the mechanism of catalase action will have to be re-examined in the near future.

In the discussion which follows the distinction between the hypothetical Michaelis compound and the recently reported catalase-hydrogen peroxide intermediate compound must be remembered (see bottom of p. 691).

Between 1 and 100 mM substrate concentration the value of k_1 is constant; and no saturation effect is observed as is required by the Michaelis theory. Although this conclusion is at variance with the results obtained by previous workers, a re-examination of their data clearly shows that the maxima in the activity-substrate relationships that they obtained fitted the Michaelis theory over only a very narrow range of substrate concentration; the data are affected by variable inactivation of the enzyme by its substrate.

A most convincing proof of the independence of k_1 and substrate concentration is given by the fact that this relation obtains at both 2 and 22° C.

The absence of any saturation affect in the activity of strong catalase had been found in 1940 in experiments conceived and largely carried out by the late G. A. Millikan. Due to the lack of independent confirmation and the imminence of the war, the data remained unpublished until now.

Independent experiments [1] show that the catalatic activity is appreciable when the substrate concentration is very small-roughly equal to that of the peroxidatic intermediate compound. The value of k_1 under these conditions is calculated to be about 1.2×10^7 liter $\times$ mole^{-1} $\times$ second^{-1}. This indicates the tremendous range of substrate concentration over which k_1 is nearly constant — from about 1×10^{-6} to 0.1 M.

Table 7. Platinum electrode experiment $[H_2O_2] = 0.3$ *mM* ($T = 25°C$).

Type of catalase	L	B	L	B	L	B	L	B	L	B	L	B
Concentration-$M \times 10^9$	2.24	1.69	5.6	4.23	11.2	8.45	4.48	2.38	2.24	1.69	1.32	0.994
Average t ½-sec	8.6	10	4.6	5.6	3.0	3.6	6.8	8.7	14.3	13.2	22	27.8
$k\frac{0.693}{(t\ ½)}$ sec^{-1}	0.081	0.070	0.15	0.12	0.23	0.19	0.10	0.08	0.049	0.053	0.030	0.025
$(k_1 = k/e) \times 10^7$-liter $\times$ mole^{-1} $\times$ second^{-1}	3.6	4.1	2.7	2.9	2.1	2.3	2.4	2.4	2.2	3.1	2.4	2.5
Ratio of activity $\frac{\text{Liver}}{\text{Blood}}$	0.87		0.93		0.90		1.0		0.70		0.96	
Average of all determinations	0.89											

These experiments do not rule out the existence of a Michaelis compound governing the catalatic activity, they merely require its lifetime to be very short. Since the error of the determinations of k_1 are less than 3 % such reactions must be at least 30 times more rapid than the observed rate of reaction of enzyme and substrate at the highest substrate concentration used (≈ 0.1 M). If only one intermediate is formed its break-down constant must exceed $30 \times 0.1 \times 3 \times 10^7$ s^{-1} or about 1×10^8 s^{-1}. Furthermore if the proposed mechanism involves a reaction with a second molecule (such as oxygen) this must

proceed with a second-order rate constant in excess of about 1×10^8 liter $\times$ mole^{-1} $\times$ second^{-1}. While the collision theory indicates that such a rate of reaction is possible, it is much more rapid than the reaction of oxygen and myoglobin. The rate of such a reaction with oxygen would have to be several orders of magnitude more rapid if catalase activity is to have no appreciable induction period when the ratio of substrate to oxygen concentration is large.

The recent data of Brdicka and Wiesner [33] on the rate of reaction of ferrohemin and hydrogen peroxide at the surface of the dropping mercury electrode give a very large value ($\approx 10^{11}$ liter $\times$ mole^{-1} $\times$ second^{-1}). Although neither the oxidation nor complex formation of ferrohematin with hydrogen peroxide has been proposed as a part of the catalase mechanism, it is interesting that their value of the reaction velocity constant for ferrohemin and hydrogen peroxide is large enough to fulfil the requirements above.

The energy of activation for the catalatic activity obtained from older methods has been found to be several fold too large since an appreciable portion of the activity was probably carried by partially inactivated enzyme. The new method gives 1700 ± 100 cal corresponding to 6 % effective collisions of enzyme and substrate. Minimum values for the energy of activation in the reactions of hemoglobin and oxygen and peroxidase and hydrogen peroxide are somewhat smaller than this. Further studies with catalase may give similarly small values if catalase inactivation is completely eliminated. if all collisions were effective, a maximum velocity constant of $\frac{3.5 \times 10^7}{0.06}$ $= 6 \times 10^8$ would be obtained.

A recalculation of the theoretical reaction velocity on the basis of these data shows that the smallest reasonable theoretical value considerably exceeds the observed rate and may be several orders of magnitude greater. This indicates a lack of data on orientation effects that may obtain in the union of enzyme and substrate and a certain indefiniteness of the theory of collisions in solutions.

Moelwyn-Hughes [34] in discussing a chain mechanism for catalase action found it difficult to reconcile a chain mechanism with an experimental value of the reaction velocity which was not in excess of the theoretical value — a chain mechanism is clearly indicated where the experimental rate clearly exceeds that possible on the basis of collisions of enzyme and substrate. Many other more obvious characteristics of the catalase reaction rule against a chain reaction:

1. The simple first-order kinetics.
2. The lack of any measurable induction period in the disappearance of substrate.

3. The apparently normal inhibition characteristics of the catalase reaction; there is agreement between the spectrophotometric and the activity data for the inhibition of catalase activity by cyanide (35).
4. Catalase has no comparable sensitivity towards »chain breakers».

On the other hand there is no evidence comparable with that obtained with peroxidase [27] to show that the rate of oxygen production is equal to the rate of disappearance of hydrogen peroxide.

Since the reaction of catalase and hydrogen peroxide follows a bimolecular course over such wide ranges of enzyme and substrate concentrations, the complex mechanisms, chain theories, Michaelis theories, ferri-ferro cycle theories, do not find support in these data. In fact the only direct evidence available that the activity of uninhibited catalase involves complexity is the existence of the intermediate compound — a topic which is to be considered elsewhere.

At pH = 3.5 the activity of catalase extrapolated back to the first few seconds of the reaction appears to be roughly equal to that at pH = 7. The principal effect of the acid solutions is to hasten the inactivation of catalase by its substrate. The latter effect also appears to be caused by acetate at pH = 7.0. The results obtained with formate where there is very little inactivation of catalase during the titration substantiate the data of Agner and Theorell [28] on anion inhibition of catalase activity.

The theory of Michaelis and Peckstein [36] that catalase activity was closely related to its amphoteric properties was greatly weakened by Agner and Theorell's finding [28] that these effects were largely due to anion inhibition and has been given a coupe de grace by these data which show that the residual pH effect found by Agner and Theorell was largely due to enzyme inactivation during the course of the reaction. The inactivation of catalase can be increased by excessively dilute enzyme, high substrate concentration, low pH, presence of acetate, etc., shaking, exposure to large surface areas, etc. and the type of inactivation seems similar in these cases. It is felt that these are factors which influence the conversion of catalase to a less active form and the factors may not in themselves represent specific effects. Although catalase has been termed »a rather rugged enzyme»[18], its maximal activity towards hydrogen peroxide is very fragile in dilute solutions.

It appears that the relative activities of the liver and blood catalase preparations are very nearly equal to the relative protohematin content when determined by methods which involve splitting the hemin from the catalase (hemochromogen, CO-hemochromogen, and pyridine hemochromogen). On the other hand, magnetometric measurements of the amount of horse-liver catalase hematin that can combine with cyanide give a result which is about 10 %

less than the ratio of the activities. In either case the data support the conclusion of Lemberg and Legge that the activity increases as the bile pigment content decreases. These data are not sufficiently extensive to prove that all hematins participate equally in the destruction of substrate.

If Kat. F. of horse-liver catalase is calculated as a »complete» catalase as recommended by Keilin and Hartree[10] the values of Lemberg and Legge[29], Sumner *et al.*[32], Keilin and Hartree[10] and Bonnichsen[15] all lie between 52,000 and 60,000 — a remarkably good agreement in view of the different methods of preparation, determination of hematin iron or bile pigment content, and extrapolation of the Kat. F. determination. This method gives a Kat. F. or erythrocyte catalase of 61,000. Over a small range, the Kat. F. (or better, the k_1) of a horse liver catalase may be calculated from this last figure by multiplying by the relative hematin iron content.

When both velocity constants are calculated on the basis of catalase molarity the value of k_1 for horse-blood catalase (3.5×10^7 liter $\times$ mole^{-1} $\times$ second^{-1}) at 22° C does indeed bear a striking resemblance to the rate of formation of the peroxidatic intermediate compound (3×10^7 liter $\times$ mole^{-1} $\times$ second^{-1})[1] and thus the statement that the latter compound forms at a rate sufficient to carry the entire catalatic activity is justifiable. It is, however, unlikely that only one hematin carries the entire activity because the data above indicate that all the catalase hematins may participate in the destruction of hydrogen peroxide. A determination of the exact relationship between the intermediate compound and the catalatic activity awaits further tests.

SUMMARY AND CONCLUSIONS

1. A modified method for the determination of catalase activity is proposed in which the enzyme concentration is increased about 50 fold over that used previously.

2. With this method the first-order reaction velocity constant is nearly independent of time, linear with enzyme concentration, independent of substrate concentration at least up to 0.1 M, and only slightly affected by temperature.

3. In order to avoid confusion with activities determined by older methods and to institute proper units, the activity is calculated from the equation $k_1 = \frac{2.3 \log_e \frac{x_0}{x}}{et}$ (see Eq. 2) instead of in terms of Kat. F. $k_1 = 3.5 \times 10^7$ and 3.0×10^7 liter $\times$ mole^{-1} $\times$ second^{-1} for horse-blood and liver catalases respectively at 22° C.

These values correspond to a Kat. F. of 6.1 and 5.2×10^4 at 0° C. For these two catalases the activity test is used for determining the enzyme concentration and the purity is expressed as the percentage of the enzyme in the total dry weight. For the purification of catalases of unknown properties, this titration procedure is recommended and the results are calculated on a dry weight basis.

4. The velocity constants for the rate of destruction of hydrogen peroxide by catalase and for the formation of the intermediate compound are very nearly equal when both values are calculated on the basis of catalase molarity.

5. The peroxidatic intermediate compound of catalase and hydrogen peroxide does not govern the velocity of the catalatic reaction in the manner required by the Michaelis theory since the value of k_1 is independent of substrate concentration up to at least 0.1 M. The previous demonstrations of a Michaelis compound of $K_m \approx 0.025$ M are concluded to be the result of enzyme inactivation. The Michaelis theory is not, therefore, an explanation of catalase action. A mechanism postulating Michaelis compounds requires that their breakdown constants be in excess of 10^8 s^{-1} and that any oxidation-reduction reactions be extremely rapid.

6. The energy of activation of the reaction of horse blood catalase and hydrogen peroxide by these methods is 1700 ± 100 calories.

7. The fraction of effective collisions of catalase and hydrogen peroxide is large and approaches that estimated for the reactions of hemoglobin and oxygen and peroxidase and hydrogen peroxide.

8. The reaction velocity for catalase and hydrogen peroxide is considerably less than that calculated on the basis of the collision theory.

9. The simplicity of the catalase kinetics obviate the need for a chain mechanism and there are several reasons against a chain mechanism for the destruction of substrate by catalase.

10. The inactivation of catalase by acid solutions and by acetate ion is not rapid but takes an appreciable time for completion. The extent of the inactivation is greatly increased by the presence of substrate. In the initial phases of the reaction the catalase activity is nearly constant from pH = 7 to pH = 3.5.

11. Excessive catalase dilution, large substrate concentration, low pH, rough treatment, the presence of acetate ion, etc. are factors which may accelerate the inactivation of catalase during the decomposition of hydrogen peroxide and may influence the conversion of catalase to a less active form.

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