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PEROXIDATIC ACTIVITY OF CATALASE

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SUMMARY

1. The oxidation of ethanol by a mixture of H_2O_2 and catalase has been studied. By using a $1 \cdot 10^6$-fold excess of ethanol (H_2A) over H_2O_2, the competition between ethanol and H_2O_2 for the active enzyme–substrate complex (C_I) has been demonstrated.

2. Under conditions where ethanol competes for C_I, the overall second-order rate constant (k_H) becomes a function of the ratio of the concentrations of ethanol and H_2O_2 (R). An increase of R causes a decrease of k_H.

3. From the dependence of k_H on R the individual rate constants of the peroxidatic mechanism of catalase action have been calculated.

$$E + H_2O_2 \xrightarrow{1} C_I \qquad k_1 = (5.6 \pm 0.5) \cdot 10^6\ M^{-1} \cdot sec^{-1}$$

$$C_I + H_2O_2 \xrightarrow{4} E + O_2 + 2\ H_2O \qquad k_4 = (1.2 \pm 0.1) \cdot 10^7\ M^{-1} \cdot sec^{-1}$$

$$C_I + H_2A \xrightarrow{5} E + A + 2\ H_2O \qquad k_5 = (1.8 \pm 0.4) \cdot 10^2\ M^{-1} \cdot sec^{-1}$$

4. From these results a steady-state saturation of 30% of catalase hematins by H_2O_2 during the catalatic reaction has been calculated.

INTRODUCTION

The essential feature of the peroxidatic mechanism of catalase action is the competition between H_2O_2 and acceptors, such as ethanol, for the active catalase $-H_2O_2$ complex (C_I) (ref. 1). A quantitative study of this competition, however, has not been made. The formation of aldehyde in a system containing catalase, ethanol and H_2O_2 (supplied continuously at a low concentration) has been demonstrated by KEILIN AND HARTREE[2]. The effect of ethanol on the disappearance of C_I has been studied spectrophotometrically by CHANCE[3–5]. In the latter experiments the concentration of free H_2O_2 was practically zero, *i.e.* measurements commenced after the

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catalatic reaction was over. Competition between H_2O_2 and ethanol had thus been virtually eliminated.

By using a $1 \cdot 10^5$–$1 \cdot 10^6$-fold excess of ethanol over H_2O_2, the actual competition between H_2O_2 and ethanol for the active intermediate can be observed. In addition, the analysis of the experimental results allows the calculation of the individual rate constants of the peroxidatic mechanism of catalase action.

EXPERIMENTAL

Materials

Catalase. Twice-crystallized beef liver catalase of Sigma was subjected to further purification. The commercial crystalline suspension was dissolved in a 20-fold excess of cold 0.01 M phosphate, pH 7. Catalase was precipitated by adding 40% (w/v) $(NH_4)_2SO_4$. After centrifugation, the colorless supernatant was discarded, and the precipitate was dissolved in a small amount of water. It was dialyzed for several days against distilled water in a cold room until a crystalline precipitate appeared. The crystals were collected and dissolved in a few drops of 5% ammonia. After the removal of insoluble residues, the solution was dialyzed for another 2 days against 0.01 M phosphate. The solution was then passed through a Sephadex G-200 column. 2-ml fractions were collected. The purity index of the fraction used as a catalyst was 0.92. (The purity index is defined as the ratio of the absorbance at 405 and 275 nm). The concentration of catalase was determined from the absorbance at 405 nm. The extinction coefficient of $\varepsilon = 340\ cm^{-1} \cdot mM^{-1}$ was used. Beef liver catalase contains 3 hematin groups. Thus the concentration of hematin is always 3 times as high as that of catalase itself. The enzyme decomposed H_2O_2 with a second-order rate constant of $k_1' = 2.3 \cdot 10^7\ M^{-1} \cdot sec^{-1}$ (in catalase units). 35–40 μM stock solutions were kept in a refrigerator for several months without a loss of activity. 20-times-diluted solutions from the stock which were directly used for kinetic experiments retained their full activity for over a month. A single dilution of catalase was used for all experiments in a series at a constant concentration of alcohol and varying concentrations of H_2O_2.

H_2O_2. Appropriate dilutions of 30% H_2O_2 (certified A.C.S.) of Fisher were used. Other materials were of analytical grade.

Methods

20 ml buffered ethanol solution were introduced into an erlenmeyer flask. 100 μl of H_2O_2 were added and the mixture was rapidly stirred with a magnetic stirrer*. The reaction was then initiated by plunging into the mixture 10 μl of the catalase solution placed on the flattened end of a glass rod. (Catalase was kept in a vial standing in an aluminum block surrounded by crushed ice.) After the lapse of a suitable time interval, the reaction was terminated by injecting 1 M trichloroacetic acid from a 2-ml syringe from which the metal needle had been removed (to prevent contamination of the quenching solution). Calibration with water showed that in this way 2.01 ml solution could be transferred within 0.2 sec with a precision of $\pm$ 0.05%. Aliquots of the quenched reaction mixture were withdrawn for the determination of H_2O_2. The

* In experiments with 1 mM H_2O_2, 10-ml solutions of buffered H_2O_2 and ethanol were mixed just prior to initiating the reaction.

reaction was then repeated by varying the reaction time. A kinetic run consisted of 7–8 independent experiments.

H_2O_2 was determined in a concentration range extending from 0.5 μM to 5 mM, in the presence of a large excess (up to $1 \cdot 10^6$-fold) of ethanol. In the mmolar range H_2O_2 was determined by iodometric titration. 0.5 g $NaHCO_3$ had been added to the solution (containing excess acid) to provide an atmosphere of CO_2 during the titration. The titration flask was sealed with parafilm and the cover was punctured with the tip of the burette. A few drops of 3% $(NH_4)_2MoO_4$ were used as a catalyst. 0.1 mM and less H_2O_2 were determined spectrophotometrically[6]. The H_2O_2 solution was added to a vigorously stirred solution of 10 mM $(NH_4)_2Fe(SO_4)_2$ in 1:4 H_2SO_4. This solution is very stable. The solid salt contains a trace of Fe^{3+}, but in the absence of H_2O_2 no additional oxidation of Fe^{2+} occurs during several hours. The solution was prepared freshly every half day. The Fe^{3+}, formed by the addition of H_2O_2, was determined by adding KSCN and by measuring the absorption at 480 nm on a Zeiss M4 QII spectrophotometer.

TABLE I

Range of $[H_2O_2]$ *determined* (μM)	*0.01 M* Fe^{2+} *added* (*ml*)	H_2O_2 *added* (*ml*)	*2.5 M KSCN added* (*ml*)	*Final vol.* (*ml*)
100 –500	5 (+30 ml H_2O)*	2	4	50
10 – 50	2	10	2	25
1 – 5	2	10	2	25
0.5– 2.5	2	10	1	13

* Improved linearity was obtained by diluting the Fe^{2+} solution with water prior to the addition of H_2O_2.

Two factors limit the sensitivity of this determination.

(1) Fe^{3+} is complexed by phosphate used as a buffer. This interference was reduced by increasing the concentration of KSCN (0.2 M in the final mixture). Calibration curves were obtained at the same concentration of phosphate as were used in the kinetic experiments.

(2) Fe^{2+} induces the oxidation of ethanol by H_2O_2 and thus reduces the amount of Fe^{3+} formed. This side reaction was minimized by adhering to the conditions summarized in Table I. For each concentration of ethanol a separate calibration curve was taken*. For 0.1 mM and 10 μM H_2O_2 the absorption was measured in a 1-cm cell and a single blank solution for all determinations was sufficient. For 1 μM H_2O_2 a 5-cm cell was used. Because of the increased absorption of the blank solution (due to its Fe^{3+} content), it was run simultaneously with each determination. The calibration curves thus obtained were mostly linear and very reproducible. The error in the determination of H_2O_2 was 1–2%.

* For 1 M ethanol and 0.1 mM H_2O_2 the order of addition of reagents was reversed: H_2O_2–H_2O–Fe^{2+}.

RESULTS

All experiments were performed at pH 7.0 in 0.067 M phosphate buffer. The temperature was 24 ± 1°. The concentration of H_2O_2 investigated extended from 0.8 to 5 mM. The concentration of ethanol varied between 0.25 M and 1 M. Catalase concentration was held virtually constant at 0.8–0.9 nM.

Details of a typical experiment are shown in Table II. The symbol $_0$ denotes initial concentration.

TABLE II

$[Catalase]_0 = 0.92$ nM; $[ethanol]_0 = 1.00$ M; temp., 24°; pH 7.0.

t (sec)	*Absorbance* (cm^{-1})	$[H_2O_2] \times 10^5$ (M)	$6 + \log [H_2O_2]$
0	0.364	2.58	1.412
10	0.314	2.21	1.344
20	0.276	1.94	1.288
30	0.239	1.66	1.220
45	0.185	1.27	1.104
60	0.151	1.03	1.013
80	0.112	0.76	0.881

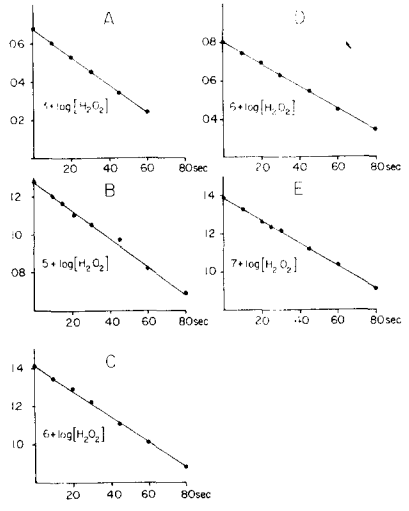


Fig. 1. First-order plots of the disappearance of H_2O_2. $[Ethanol]_0 = 1.00$ M; pH 7.0; temp., 24°.

	$[Catalase]_0$ (nM)	$[H_2O_2]_0$ (μM)
A	0.925	4750
B	0.920	181
C	0.920	25.8
D	0.920	6.33
E	0.920	2.40

TABLE III

Temp., 24°; pH 7.0.

0.25 M ethanol		*0.50 M ethanol*		*1.00 M ethanol*	
$[H_2O_2]$ (μM)	$k_H \times 10^{-6}$ ($M^{-1} \cdot sec^{-1}$)	$[H_2O_2]$ (μM)	$k_H \times 10^{-6}$ ($M^{-1} \cdot sec^{-1}$)	$[H_2O_2]$ (μM)	$k_H \times 10^{-6}$ ($M^{-1} \cdot sec^{-1}$)
4500	7.34	4610	6.40	4750	6.20
291	7.10	310	6.40	181	6.02
26.4	6.57	296	6.43	25.8	5.56
6.30	6.17	25.5	5.94	6.33	4.85
2.58	5.70	5.10	5.40	2.40	4.83
		2.50	5.24		
		1.36	4.93		

First-order plots of a series of experiments at constant ethanol concentration and varying initial concentrations of H_2O_2 are shown in Fig. 1 (the data of Table II included). Second-order rate constants on hematin basis, k_H, were calculated by dividing the first-order rate constants by $3 \cdot [Cat]_0$. Values of k_H obtained at different initial concentrations of ethanol and H_2O_2 are summarized in Table III.

log k_H as a function of log $\bar{R}$ (the average ratio of the concentrations of ethanol and H_2O_2) is plotted in Fig. 3.

DISCUSSION

Our discussion is based on the peroxidatic theory of catalase action.

Scheme A

$$E + H_2O_2 \xrightarrow{1} C_I$$

$$C_I + H_2O_2 \xrightarrow{4} E + O_2 + 2\,H_2O$$

$$C_I + H_2A \xrightarrow{5} E + A + 2\,H_2O$$

E denotes catalase hematin. H_2A stands for an acceptor (ethanol).

The rate law for the disappearance of H_2O_2 in the steady state is given by:

$$-\frac{1}{[E]_0}\frac{d\ln[H_2O_2]}{dt} = k_H = k_1\frac{2k_4 + k_5R}{(k_1 + k_4) + k_5R} \qquad (1)$$

$R = [H_2A]/[H_2O_2]$; k_H is a function of R. For $k_4 \gg k_5R$, k_H becomes $2/[(1/k_1) + (1/k_4)]$ which is the usual rate constant for the catalatic decomposition of H_2O_2. If $k_4 \ll k_5R$, k_H is equal to k_1.

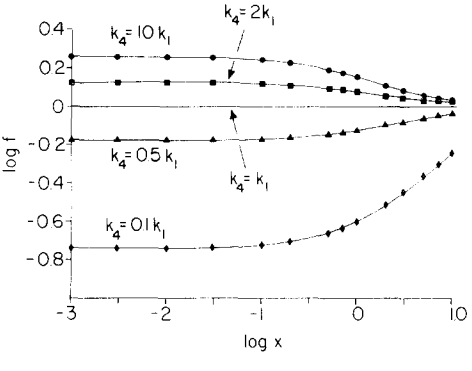
Thus the study of the overall rate of disappearance of H_2O_2 provides a method for calculating individual rate constants of the peroxidatic mechanism of catalase action.

In order to observe significant changes of k_H, a substantial fraction of H_2O_2

must be consumed during the reaction in the oxidation of the acceptor. In addition, this fraction should vary both below and above 0.5. In view of the high reactivity of C_I with H_2O_2, this condition requires that the acceptor should be in a large excess over H_2O_2. This is achieved by reducing the concentration of H_2O_2 to 1 μM.

According to Eqn. 1, k_H can increase or decrease by increasing R, depending on whether k_1 is greater or less than k_4. For $k_1 = k_4$, k_H becomes independent of R. An inspection of Fig. 3 reveals that for beef liver catalase $k_4 > k_1$.

A family of curves: $f = (2 + x)/\{1 + (k_1/k_4)\} + x$ have been calculated for different ratios of k_1 and k_4. A selection of them is shown on a logarithmic scale in Fig. 2. f and x are related to k_H and R by the equations: $k_H = k_1 f$ and $R = (k_4/k_5)x$. Thus the log k_H *vs.* log R curves can be superimposed directly on the log f *vs.* log x curves by applying the coordinate shifts log k_H and log (k_4/k_5). Pairs of values of log k_H and log R have been calculated from the data of Table III and are plotted in



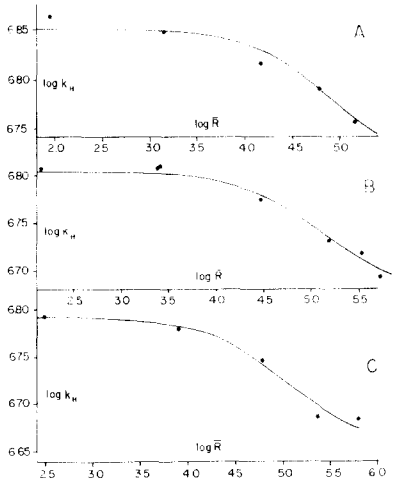


Fig. 2. Calculated curves of log f.

Fig. 3. log k_H as a function of log $\bar{R}$. pH 7.0; temp., 24°; $[Ethanol]_0$ = 0.25 M (A), 0.50 M (B), 1.00 M (C).

Fig. 3 together with the best-fitting curves. For R (the changing ratio of the concentration of ethanol and H_2O_2 during the reaction) its average value $\bar{R}$ has been substituted. For the purpose of calculation the reaction time has been limited to the first half-life. The parameters of the best-fitting curves are summarized in Table IV. The individual rate constants are given in Table V. The value of k_1 varies with the concentration of alcohol. Its limiting value in pure aqueous solution is $(5.6 \pm 0.5) \cdot 10^6$ $M^{-1} \cdot sec^{-1}$. It has been calculated from $k_H = 7.65 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$ (obtained in the absence of ethanol) taking $(k_4/k_1) = 2.2$. The corresponding value of k_4 is $(1.2 \pm 0.1) \cdot 10^7$ $M^{-1} \cdot sec^{-1}$.

The accuracy in the determination of k_5 is low. It is due to the very low reactivity of ethanol towards C_I and to the inability to extend the measurements to even higher concentration ratios of ethanol and H_2O_2. The average value of k_5 from Table V is $(1.8 \pm 0.4) \cdot 10^2$ $M^{-1} \cdot sec^{-1}$.

TABLE IV

Temp., 24°; pH 7.0.

$[Ethanol]_0$ (M)	k_4/k_1	$\log k_1$	$\log (k_4/k_5)$
0.25	2.40	6.704	4.70
0.50	2.05	6.676	4.86
1.00	2.20	6.654	4.80

From our rate constants a very nearly 30% saturation of catalase hematins is calculated in the steady state. This value is in fair agreement with previous estimates of this ratio. However, the basis of these calculations must be examined.

All methods for the calculation of individual rate constants of the mechanism of catalase action are based on the measurements of $k_H = 2/\{(1/k_1) + (1/k_4)\}$ and of another quantity composed of k_1 and k_4.

One method has been based on the measurement of the relative changes of

TABLE V

Temp., 24°; pH 7.0.

$[Ethanol]_0$ (M)	$k_1 \times 10^{-6}$ $(M^{-1} \cdot sec^{-1})$	$k_4 \times 10^{-7}$ $(M^{-1} \cdot sec^{-1})$	$k_5 \times 10^{-2}$ $(M^{-1} \cdot sec^{-1})$
0.25	5.06	1.22	2.4
0.50	4.74	0.98	1.4
1.00	4.51	0.99	1.6

absorbance accompanying the formation of the primary catalase–H_2O_2 and catalase–methylhydrogen peroxide (ethylhydrogen peroxide) complexes[3,7]. On the assumption that these complexes have identical extinction coefficients at 405 nm, and that the primary alkylhydrogenperoxide complexes are formed quantitatively in an excess of the respective alkylhydrogenperoxides, a 22% saturation of catalase hematins by H_2O_2 has been calculated. This value does not differ substantially from the 30% calculated above. However, the assumed identity of the extinction coefficients cannot essentially be proven.

An alternative procedure has been based on the measuring of the extent of binding of cyanide by a reaction mixture of catalase and H_2O_2 (ref. 7). The differential absorbance of a mixture of catalase, cyanide, and H_2O_2 against a pure catalase solution of equal strength (at a wavelength where catalase and the catalase–H_2O_2 complex are isosbestic) has been denoted by Δ_2. The differential absorbance of an identical solution of catalase and cyanide but without H_2O_2 against pure catalase is denoted by Δ_1. The degree of saturation of catalase hematins by H_2O_2 in the presence of cyanide is then given by:

$$\frac{[C_1]_2}{[E]_0} = 1 - \frac{\Delta_2}{\Delta_1} \frac{[CN^-]_0 - [ECN^-]_1}{[CN^-]_0 - [ECN^-]_2} \frac{[E]_1}{[E]_0} - \frac{[ECN^-]_2}{[E]_0} \qquad (2)$$

The subscripts 1 and 2 denote the two experiments referred to above*. The derivation is based on a simple equilibrium between catalase and cyanide:

$$E + CN^- \underset{}{\overset{K_i}{\rightleftarrows}} E\,CN^-$$

It should be noted that Eqn. 2 is valid regardless of whether or not CN^- disturbs the established steady state between catalase and H_2O_2. If CN^- does not disturb the catalase–H_2O_2 steady state, as assumed previously[7,8], a constant value

TABLE VI

$[E]_0 = 3.4\ \mu M$ horse liver catalase; $[H_2O_2]_0 = 10\ \mu M$; $K_i = 4\ \mu M$. Data of CHANCE[7].

$[CN^-]_0$ (μM)	$\frac{[C_I]_2}{[E]_0}$	$1 - \frac{A_2}{A_1}$	$[CN^-]_2$ (μM)
0.4	0.62	0.44	0.29
0.8	0.53	0.38	0.55
2.0	0.35	0.27	1.42
4.0	0.36	0.30	3.05
8.0	0.24	0.20	6.36
20	0.18	0.18	17.7
50	0.08	0.08	47.1
200	—	0.03	—

of $[C_I]_2/[E]_0$ is expected at all values of $[CN^-]_0$. The data of Table VI do not show such a behavior. If, however, CN^- displaces H_2O_2 from C_I, then $[E]_0/[C_I]_2$ should vary linearly with $[CN^-]_2$.

$$\frac{[E]_0}{[C_I]_2} = 1 + \frac{k_4}{k_1} + \frac{(k_4/k_1)}{K_i}[CN^-]_2 \qquad (3)$$

By extrapolation of $[E]_0/[C_I]_2$ to zero cyanide concentration, the degree of saturation of catalase hematins by H_2O_2 in the absence of cyanide can be evaluated. The data of Fig. 4 show a more complicated behavior than indicated by Eqn. 3. From

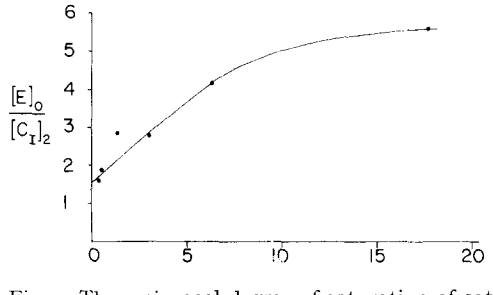


Fig. 4. The reciprocal degree of saturation of catalase by H_2O_2 as a function of the free cyanide concentration. ($[CN^-]_2$ in μM). $[E]_0 = 3.4\ \mu M$ horse liver catalase; $[H_2O_2]_0 = 10\ \mu M$. Data of CHANCE[7].

* The quantity $1 - (A_2/A_1)$ has previously been taken as a measure of $[C_I]_2/[E]_0$. This implies that the formation of ECN^- from E and CN^- is quantitative[7]. However, in view of the magnitude of K_i and of the range of $[CN^-]_0$ employed, this assumption is generally not valid. $1 - (A_2/A_1)$ and $[C_I]_2/[E]_0$ agree well at high $[CN^-]_0$. At low $[CN^-]_0$, however, $1 - (A_2/A_1)$ gives too low values for $[C_I]_2/[E]_0$.

the initial part of the curve a 65% saturation can be calculated at zero cyanide concentration. This value deviates greatly from previous estimates of this quantity and also from the value reported in this work. The reason for this discrepancy is not clear. It is possible that the system containing cyanide is more complex than assumed in this treatment.

Another relation between k_1 and k_4 can be obtained from a study of the rate of formation of C_I in the pre-steady state. Denoting the steady state concentration of C_I by $[C_I]_s$ and assuming a constant concentration of H_2O_2, $[H_2O_2]_0$, during the build-up of C_I, we obtain[1]

$$\ln \frac{[C_I]_s}{[C_I]_s - [C_I]} = (k_1 + k_4)\,[H_2O_2]_0\, t \qquad (4)$$

The respective absorbance decrements can be substituted for C_I. The knowledge of the extinction coefficient of C_I is not required. The value $k_1 + k_4 = 1.6 \cdot 10^7\ M^{-1} \cdot sec^{-1}$ has been obtained for horse erythrocyte catalase[1]. From steady state studies on the same system $k_1k_4/(k_1 + k_4) = 4.4 \cdot 10^6\ M^{-1} \cdot sec^{-1}$ has been calculated. These two data are incompatible since complex numbers for k_1 and k_4 are calculated from them. (By the way, this calculation cannot distinguish in principle between k_1 and k_4.)*

It is concluded from the preceding discussion that competitive oxidation presently provides the most reliable method for the calculation of individual rate constants. In particular, all experiments from which k_1 and k_4 have been calculated were performed using the same concentration of catalase. In all the other methods, decomposition experiments were performed using nmolar (and lower) concentrations of catalase, while a second set of experiments was performed using several μmolar concentrations of catalase. The 1000-fold gap in catalase concentration between the two sets of experiments may be partially responsible for the observed discrepancies.

The value of $k_5 = 1.8 \cdot 10^2\ M^{-1} \cdot sec^{-1}$ is considerably smaller than the accepted one of $1000\ M^{-1} \cdot sec^{-1}$ (refs. 3–5). It is to be noted that the present value was determined under experimental conditions, where C_I was involved in the decomposition of the bulk of H_2O_2. The previous one had been obtained at a very late stage of the reaction when practically all the H_2O_2 had been decomposed. For this reason, the lower value more likely represents the true rate constant of the reaction between ethanol and C_I.

DISCUSSION BASED ON THE EXTENSION OF THE PEROXIDATIC MODEL OF CATALASE ACTION

The previous discussion was based on the peroxidatic model of catalase action *(Scheme A)*. However, the results of experiments using high concentrations of H_2O_2 make an extension of this basic scheme necessary[9,10]. Several modifications have been proposed.

Mechanism B, forwarded by OGURA[9], is based on the formation of a biperoxy

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* NICHOLLS[8] calculated 18% (or 82%) saturation of horse erythrocyte catalase by H_2O_2. His calculation is based on taking $k_1 = 0.70 \cdot 10^7\ M^{-1} \cdot sec^{-1}$ from Table I of ref. 1. However, this value deviates greatly from the average and was obtained using the lowest initial H_2O_2 concentration. There seems to be no justification for this arbitrary choice of k_1.

complex of catalase. There are two variations of this scheme. In *Scheme* B_1, the biperoxy complex is assumed to be catalytically inactive.

Scheme B_1

$$E + H_2O_2 \underset{2}{\overset{1}{\rightleftharpoons}} C_I$$

$$C_I + H_2O_2 \underset{7}{\overset{6}{\rightleftharpoons}} C_I'$$

$$C_I + H_2O_2 \xrightarrow{4} E + O_2 + 2\,H_2O$$

$$C_I + H_2A \xrightarrow{5} E + A + 2\,H_2O$$

In Mechanism B_2, C_I' is assumed to be catalytically active.

Scheme B_2

$$E + H_2O_2 \underset{2}{\overset{1}{\rightleftharpoons}} C_I$$

$$C_I + H_2O_2 \underset{7}{\overset{6}{\rightleftharpoons}} C_I'$$

$$C_I' \xrightarrow{3} E + O_2 + 2\,H_2O$$

$$C_I + H_2A \xrightarrow{5} E + A + 2\,H_2O$$

In Mechanism C the existence of two monoperoxy complexes is assumed, in analogy to Fe^{3+} and hemin catalysis[11,12]. The mechanism of BRILL[13], postulating a partial oxidation of the porphyrin ring, belongs also to this category.

Scheme C

$$E + H_2O_2 \underset{2}{\overset{1}{\rightleftharpoons}} C_I$$

$$C_I \xrightarrow{3} C_{II}$$

$$C_{II} + H_2O_2 \xrightarrow{4} E + O_2 + 2\,H_2O$$

$$C_{II} + H_2A \xrightarrow{5} E + A + 2\,H_2O$$

All three mechanisms make it possible to account for the presently available experimental results. Expressions for k_H have been obtained on the basis of all three of them. These are summarized in Table VII together with their (partial) decomposition to individual rate constants. K (in B_2) $= (k_3 + k_7)/k_6$ and K_m (in C) $= (k_2 + k_3)/k_1$. The expressions are valid at low $[H_2O_2]$, where the rate of decomposition of H_2O_2 in the absence of ethanol is strictly first-order with respect to $[H_2O_2]$. Under

TABLE VII

Mechanism	k_H	*Individual rate constants*		
A	$k_1 \frac{2k_4 + k_5 R}{(k_1 + k_4) + k_5 R}$	k_1	k_4	k_5
B_1	$k_1 \frac{2k_4 + k_5 R}{(k_1 + k_4) + k_5 R}$	k_1	k_4	k_5
B_2	$k_1 \frac{(2k_3/K) + k_5 R}{(k_1 + (k_3/K)) + k_5 R}$	k_1	k_3/K	k_5
C	$(k_3/K_m) \frac{2k_4 + k_5 R}{((k_3/K_m) + k_4) + k_5 R}$	k_3/K_m	k_4	k_5

these circumstances *Scheme* B_1 degenerates into *Scheme A*. k_3/K in *Scheme* B_2 corresponds to the bimolecular rate constant of the reaction between C_1 and H_2O_2, if we assume that a ternary complex C_1' is formed between them. k_1 becomes k_3/K_m in *Scheme C*. In the special case when $k_2 \ll k_3$, $k_3/K_m = k_1$.

It is seen from Table VII that the meaning of the individual rate constants is not essentially changed in any of the extensions of the peroxidatic mechanism. In particular, k_5, the rate constant of the reaction of the active intermediate with the acceptor is obtained in the same way in all cases.

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