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An Intermediate Compound in the Catalase-hydrogen peroxide Reaction

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A review of the recent developments in the study of catalase has been made by Theorell [1]. The bulk of the past literature contains the following types of studies on the mechanism of catalase action:

1. Determination of the overall catalatic activity in very dilute enzyme-solutions by extrapolation to the activity at $t = 0$ (Euler and Josephson [2]).

2. Comparisons of the activity of inorganic catalysts with catalase in order to determine the mechanism by analogy (for a summary see Stern [3]).

3. The study of the catalytic activity model systems such as catalase-ethylhydroperoxide (Stern [4]) and azide-catalase-peroxide (Keilin [5]) leading to various conclusions concerning the activity of the enzyme in the presence of hydrogen peroxide.

4. The study of the »coupled» or peroxidatic reaction of catalase, alcohols, and enzyme systems which produce hydrogen peroxide continuously (Keilin[6]).

In no case has the activity of this enzyme been studied by means of the characteristics of a spectroscopically-defined intermediate compound of enzyme and substrate as in the case of peroxidase [7]. In fact the papers of Haldane [8] and Keilin [5] tend to discourage the search for such a compound, to quote from Keilin [5], »the reaction with H_2O_2, which consists in a violent, almost explosive, decomposition of the latter, during which it is impossible to observe any changes in colour and absorption spectrum of the enzyme».

These studies [9] were initiated in 1938 in a serious effort to find an intermediate compound of catalase and hydrogen peroxide. Due to a large number of technical difficulties success has only been achieved in the last few months and this paper gives preliminary spectral, kinetic, and equilibrum data. This intermediate compound differs markedly from the one postulated by the

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Michaelis theory and stated by Euler and Josephson [2] to have a very low affinity for H_2O_2. This compound has a high affinity for hydrogen peroxide, exists at a constant concentration during catalatic activity, and decomposes slowly in the absence of alcohols and rapidly in their presence. The compound appears to be a ferric-iron hydrogen-peroxide complex, which has many properties in common with those of peroxidase. However, this complex has both peroxidatic and catalatic properties.

METHOD

The bulk of the data presented in this paper was obtained by what may be called »Spectrophotometry with Time Resolution». The cuvettes containing solvent and solution usually employed in the spectrophotometry of liquids are replaced by a single capillary »cuvette» into which the *mixed* but *unreacted* components of the solution are rapidly injected. Modifications of the mixing chambers of Roughton and Millikan [10] are incorporated in the syringe unit of fig. 1. Time resolution requires a small bore capillary (1 mm) which can be rapidly filled with unreacted solutions. Second order reactions require dilute solutions ($\sim 1 \times 10^{-6}$ *M*) in order to reduce their rate of reaction. The effective

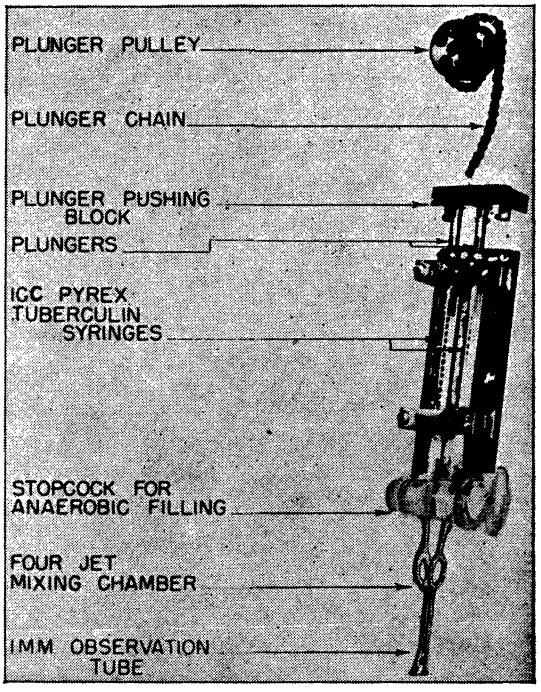

Fig. 1. The syringe unit.

depth of the capillary, as illuminated by the exit slit of the spectrophotometer, is only 0.47 mm. The density of strong enzyme solutions is, therefore, very small ($\log \frac{Io}{I} \leq 4 \times 10^{-2}$), and the density changes due to some intermediate compounds much smaller ($\log \frac{Io}{I} \approx 3 \times 10^{-3}$).

The measurement of these small density increments with an accuracy of 3 % ($\log \frac{Io}{I} \approx 1 \times 10^{-4}$), requires an extremely stable light source and photoelectric recording system. The technical details of this apparatus will soon appear elsewhere [11] and only an outline of the function is given here. As fig. 2 shows, the voltage for the tungsten lamp is stabilized by an electronic controller which reduces fluctuations by 3×10^4. The drift of the lamp voltage is less than 10^{-5} parts per minute. A double monochromator (Coleman Electric Company) covering a range from 340 to 1000 mμ and having a spectral

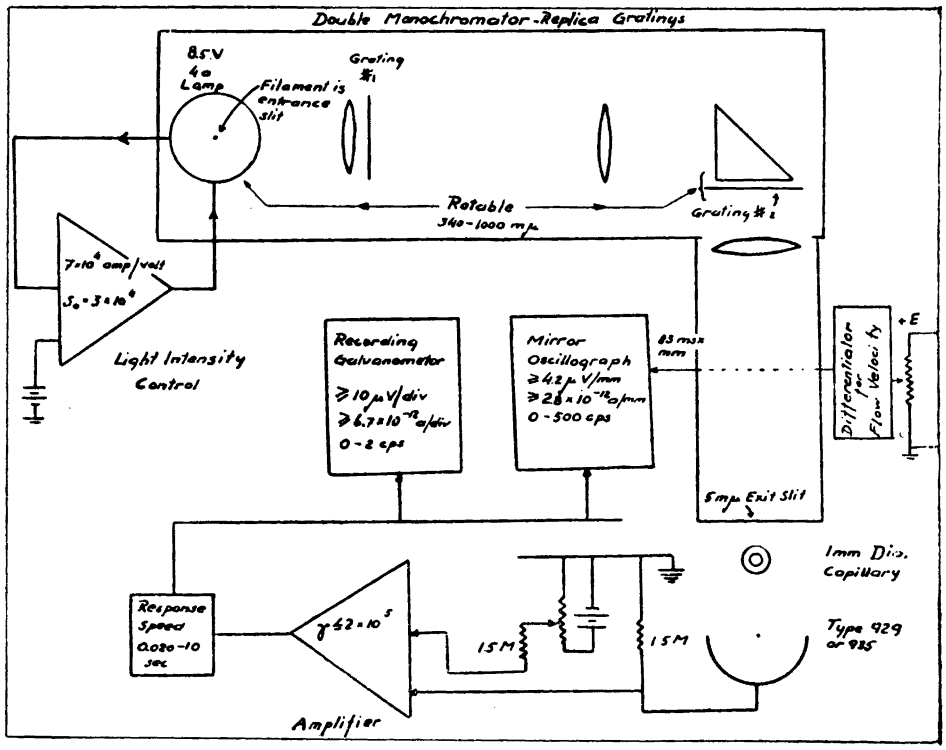

Fig. 2. Functional diagram of the method for obtaining spectroscopic data on unstable intermediate compounds.

interval of 5 mμ illuminates the photocell with light transmitted through the capillary. The photocurrent is increased in a stable amplifier of adjustable response speed and finally operates two recorders (1) an ink-writing recorder having a response to 2 cps. and (2) a photographic recorder using a galvanometer oscillograph responding up to 500 cps.

The method of operation consists simply of filling the syringes with enzyme and substrate, setting the gain, response speed, wavelength, and zero point appropriately, and pushing upon the bakelite block holding the syringe plungers together (fig. 1). The capillary is then filled with the mixed but unreacted components of the solution and, after the flow has stopped, the reaction is recorded directly in ink or upon photographic paper. This is called the »stopped flow method» (fig. 3).

If the reaction to be studied occurs in less than 0.05 s the flow method of Hartridge and Roughton [10] is employed — but no modification of the apparatus is required except, (1) the recording is done at an appropriately higher speed of photographic paper and amplifier response and (2) flow velocity is simultaneously recorded photographically. Each value of flow velocity corresponds to a certain time interval between mixing and observation and to a certain value of optical density or concentration of intermediate compound (fig. 5).

PREPARATIONS

All the catalase solutions used in these experiments were prepared and crystallized by Bonnichsen [12, 13] and the tests of a large number of different catalases (table 1) would have been impossible without his assistance. The concentration of the horse liver catalases was determined straightforwardly from $\varepsilon_{405\,(c\,=\,1\,mM)} = 340$ (Agner [14]). For the other catalases, the concentration was determined on a hematin-iron basis and, in the

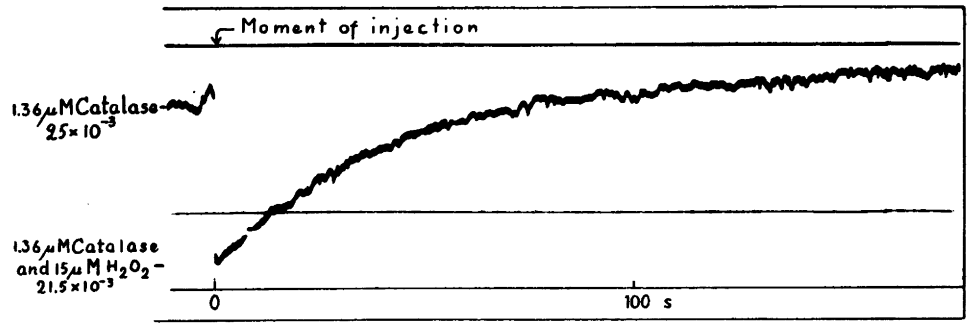

Fig. 3. The kinetics of the intermediate compound in the catalasehydrogen-peroxide reaction at 405 mμ.

case of the blood and kidney catalases where there are four hemin groups, the extinction per catalase molecule was taken as $\varepsilon_{405\,(c\,=\,1\,mM)} = 380$ (Bonnichsen [12]).

THE SPECTRUM OF THE INTERMEDIATE COMPOUND

Many, many experiments with an apparatus using glass color filters [7] and with this apparatus were fruitless because the assumption was made that the spectrum of the intermediate compound would resemble that of azide-catalase-peroxide. The first apparatus balanced 385 mμ against 420 mμ while in the second, a wavelength of 430—435 mμ was used. Also these fruitless experiments were made under optimum conditions dictated by the Michaelis theory; as dilute enzyme as feasible.

At a wavelength of 405 mμ and with a catalase concentration in excess of 1 μM and a several fold excess of H_2O_2, the transient intermediate compound of catalase and hydrogen peroxide is found and a typical record of its formation and disappearance is shown in fig. 3. At the moment of injecting catalase and hydrogen peroxide into the capillary a very small ($\log \frac{Io}{I} = 4 \times 10^{-3}$), but very rapid decrease of density occurs. The compound is momentarily stable

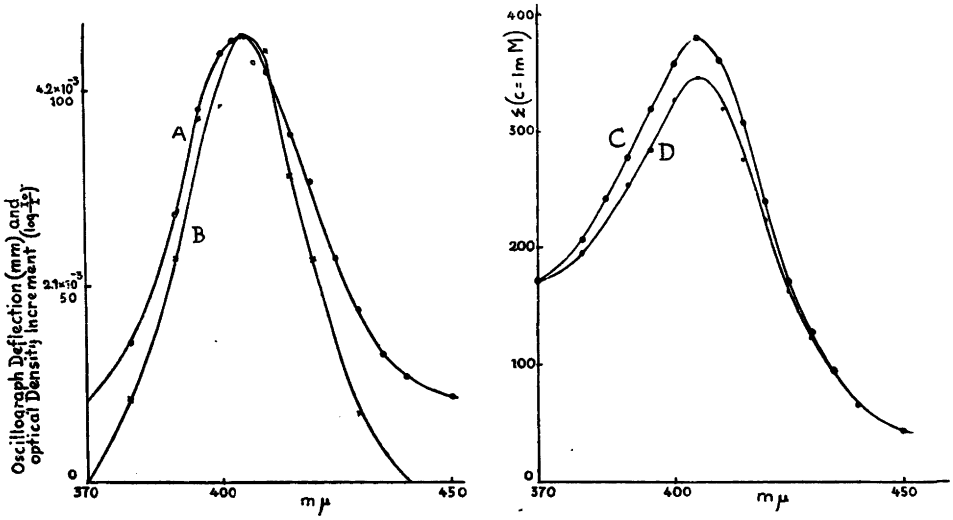

Fig. 4. The spectrum of the intermediate compound in the region of the Soret band. The left figure compares the differences between catalase and water (A) with those between catalase and the intermediate compound (B). The right figure shows an absolute spectrum computed from the difference spectrum. Expt. 51 b + d. 3.4 μM horse blood catalase, 7.5 μM H_2O_2, $S = 5$ for curve A, $S = 20$ for curve B (an eleven-fold change), pH = 6.7, 0.01 M phosphate. C is the catalase spectrum and D the catalase-hydrogen peroxide spectrum.

and then decomposes slowly liberating catalase in a reaction which requires 40 s for half completion in this experiment.

Fig. 4 (left) shows the variation of the oscillograph deflection (crosses) with wavelength. This is the »difference spectrum» between catalase and the intermediate compound over the range where the deflection exceeds the experimental error. For comparison the »difference spectrum» of the same catalase solution and water, but with the sensitivity reduced 11 times, is plotted with circles.

The difference spectrum of the intermediate compound can be converted into an absolute spectrum by applying these differences, with appropriate corrections, to a catalase spectrum determined in the ordinary manner. The following procedure is used:

1. The spectrum of a catalase solution is taken in the usual manner (Beckman spectrophotometer) and converted into units of $\varepsilon_{c=1\,mM}$.

2. This solution is used to calibrate the capillary by measuring the oscillograph deflection with respect to water — usually at 405 mμ where slight errors of wavelength have less effect.

3. At the same wavelength the oscillograph deflection corresponding to the difference between catalase and the maximum concentration of the intermediate compound is converted to absolute units by the formula

$$\Delta\,\varepsilon_I = \varepsilon_c \frac{D_k}{D_s} \cdot \frac{S_k}{S_s} \cdot \frac{C_s}{C_k}$$

where $\Delta\,\varepsilon_I$ = the difference of extinction between catalase and the intermediate at λ_1,
ε_c = the extinction of catalase at λ_1,
D_k = the maximum deflection of the oscillograph in the kinetic experiment at λ_1,
D_s = the deflection of the oscillograph in the static calibration at λ_1,
S_k = the sensitivity in the kinetic experiment (μvolts/mm),
S_s = » » » » static calibration (» / »),
C_s = » concentration » » » » (μM)
C_k = » » » » kinetic experiment (μM).

4. The value of $\Delta\,\varepsilon_I$ at $\lambda_1 \ldots n$ is obtained from the values of $(D_k)\,\lambda_1 \ldots n$ and the value of the photocurrent at the particular λ. The absorption of the catalase solution used was so small ($\log \frac{Io}{I} = 4 \times 10^{-2}$) that no corrections were made for this factor.

$$(\Delta \varepsilon_I)_{\lambda_n} = (\Delta \varepsilon)_{\lambda_1} \cdot \frac{E_{\lambda_1}}{E_{\lambda_n}} \cdot \frac{(D_k)_{\lambda_n}}{(D_k)_{\lambda_1}} \cdot \frac{(S_k)_{\lambda_n}}{(S_k)_{\lambda_1}}$$

where $E_{\lambda_1 \ldots n}$ is proportional to photocurrent (units are millivolts).

5. The values $(\Delta \varepsilon_I)_{\lambda_1 \ldots n}$ are then applied to the curve 1 above with the appropriate sign; $\varepsilon_I = \varepsilon_c - \Delta \varepsilon_I$.

The result is shown in the right-hand portion of fig. 4. The features of these data are

1. The Soret band of the intermediate compound is sharper than that of catalase.

2. It is shifted a few millimicrons to the visible (see left figure).

3.* The extinction coefficient is only slightly less than that of catalase ($\sim$ 90 %).

4.** The spectrum does not resemble those of the catalase-cyanide or azide-peroxide compounds.

Using even stronger catalase (7.5 μM) some deflections are obtained between 500 and 550 mμ ($(\Delta \varepsilon_I)_{\lambda = 530} \approx 5$) and have values approximately equal to the minimum detectable at the Soret band (at 425 mμ for example). The absence of spectral data in the intervals 430—490 mμ and 560—650 mμ is, therefore, no convincing proof that such data do not exist. It is hoped that a refinement of the method will give the isobestic points and the complete spectrum; at present, the data are apparently only obtainable when the difference between the absorption of catalase and the intermediate compound is large.

Table 1. The extent of the spectral shift at 405 mμ for various catalase preparations.

Type	Relative catalase activity	$\frac{D_{405}}{D_{280}}$	Conc. (μM)	$\Delta \varepsilon_{405}$	$\left(\frac{\Delta \varepsilon}{\varepsilon}\right)_{405}$
Horse blood	1***	1.2	1.9	42	0.11
Horse blood	—	0.96	4.3	32	0.08
Horse kidney	0.65	1.0	1.6	39	0.10
Horse liver	—	1.0	3.3	36	0.11
Horse liver	0.52	0.38	1.9	29	0.09
Horse liver	0.28	0.46	2.3	16	0.05
Human liver	0.31	0.7	3.4	19	0.06

* It is interesting to note that this is nearly equal to the difference between the extinction of three and four hemin catalases [12].

** A graphic illustration of the difference between the spectrum of the intermediate compound and that of the cyanide compound is given in fig. 22.

*** Cat. F. $\approx$ 60,000.

A large number of different catalase preparations gave similar values for $(\Delta \varepsilon_I)_{\lambda = 405}$ and the characteristics of the preparations and the spectral data are tabulated in table 1. In the last two preparations a somewhat smaller concentration of the compound was formed. This may possibly be correlated with the relative catalatic activity.

THE FORMATION OF THE INTERMEDIATE COMPOUND

Fig. 5 shows a 100-fold time expansion of the initial decrease of density of fig. 4. Here the records clearly show that the compound does not completely form until the flow stops — a time delay of nearly 30 milliseconds. In fact the compound is roughly half formed in about 8 milliseconds. Other records taken with larger concentrations of H_2O_2 show the intermediate compound to be completely formed at the earliest time after mixing (fig. 18 and 23).

The three experiments shown in fig. 5 were taken under widely different conditions, in A the compound is stable for about a second after its formation;

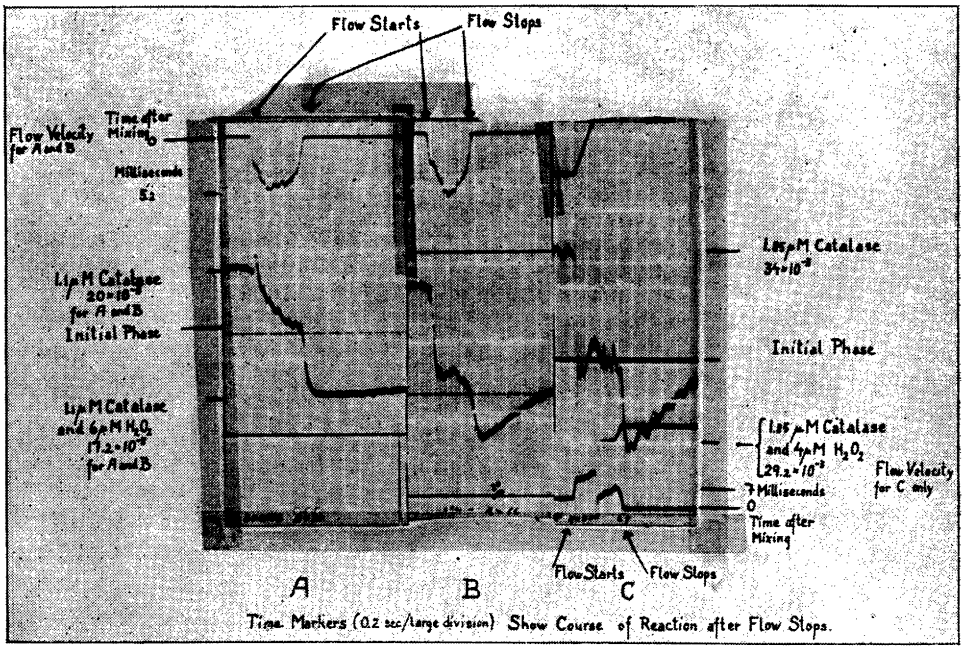

Fig. 5. The kinetics of the formation of the intermediate compound. Curves A and B were obtained with the apparatus of fig. 2 while C was obtained with an apparatus using a Jena UG — 3 filter. Ethanol was added in B and C (400 and 500 μM respectively). Time markers (0.2 s/large division) show course of reaction after flow stops.

in B and C it decomposes more rapidly due to added alcohol. This effect will be discussed later. Curves A and B were obtained using a monochromator while C was obtained in a different apparatus using a Jena-UG 3 filter and a more rapidly responding amplifier.

A second order velocity constant* was obtained from curves similar to C and the values are listed in table 2.

Table 2. Experiments 69 and 75.

H_2O_2 — μM	2	4	8	10	20
$k_1 \times 10^{-7}$ L $\times$ $M^{-1} \times s^{-1}$	7.0	2.4	3.1	3.8	1.4
e	1.85 μM horse blood catalase				
a	1000 μM ethyl alcohol				

The deviations of the values for $H_2O_2 = 2$ and 20 do not permit the statement that the reaction strictly obeys a second order equation over the entire range although the data for $H_2O_2 = 4$, 8, and 10 strongly favor this viewpoint and in fact are much more reliable for the following reasons:

1. At 20 μM the reaction is 85 % complete at the earliest time measured.
2. At 2 μM the amount of intermediate formed is 1/3 the saturation value and the errors are increased proportionally.

For the middle concentrations the value of 3×10^7 may be compared with that for peroxidase, myoglobin, and the calculated value for catalase, 1.2 (Chance [7]), 1.9 (Millikan [15]), 0.76 (Haldane [8]) $\times 10^7$ respectively and it is seen to be somewhat larger, especially with respect to the calculated value. This is an extremely important comparison since it reveals that *the rate of formation of this compound is sufficient to carry the entire catalatic activity of this enzyme* providing a mechanism for its rapid decomposition is afforded. The exact value of the velocity constant and the determination of the range over which a second order equation holds awaits further experiment.

THE DECOMPOSITION OF THE INTERMEDIATE COMPOUND

The experimental data, however, show that the decomposition of this intermediate compound is an extremely slow first-order reaction as is illustra-

* In this calculation it is assumed that the intermediate compound consists of one molecule of H_2O_2 per catalase molecule. On the basis of four H_2O_2 per catalase molecule the velocity constant would be somewhat larger.

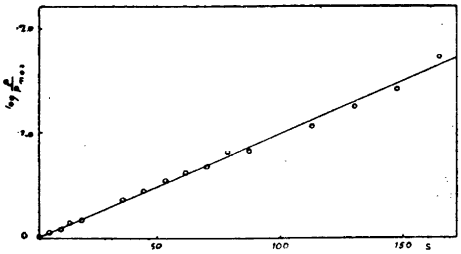

Fig. 6. The order of the reaction for the decomposition of the intermediate compound. The curve of fig. 3 plotted according to the first order equation, $k_3 = 0.02\ S^{-1}$. Expt. 59, 1.36 µM horse blood catalase, 12 µM H_2O_2, $\lambda = 405$, $S = 30$, $t = 24°\ C$, $pH = 6.7$, 0.01 M phosphate.

ted by fig. 6 where the data of fig. 3 are analysed. The reaction velocity constant is 0.02 S^{-1}. This decay time is much too long to account for the high catalatic activity of the enzyme. Furthermore the decay time and duration of the »cycle» do not in any way vary in the manner found experimentally and theoretically for the enzyme-substrate compound of peroxidase [13].

$$t_{1/_2\ \mathrm{off}} = \frac{x_0}{p_{\max} k_3}$$

Further support to this discrepancy is afforded by the data of fig. 7 where the time from the maximum to half the concentration of the intermediate is shown to be relatively *independant* of the peroxide concentration — certainly not proportional to it as the equation above requires.

These data also show another characteristic of the decay of the compound — considerable variation from preparation to preparation. In no case has the compound been completely stable and in cases where the compound decayed rapidly, a 24 hour dialysis removed the alcohol in the preparation and the usual rate was obtained — on the average about 40 s half time. In a particular case a preparation was repeatedly dialysed and then dried in order to remove all traces of alcohol. The usual rate was, however, obtained. The rate of decomposition is temperature sensitive and some of the systematic

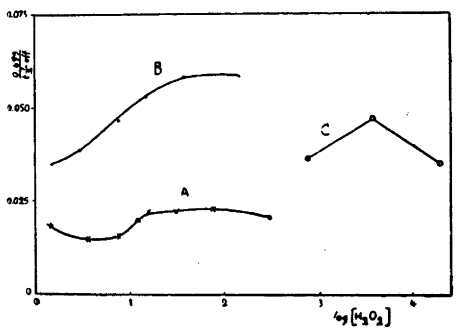

Fig. 7. The effect of initial hydrogen peroxide concentration upon the rate of decomposition of the intermediate compound.

× *Expt. 50 b, 0.68 µM horse blood catalase.*
• *Expt. 51 a, 3.4 µM » » »*
○ *Expt. 57, 1.36 µM » » »*
△ *Expt. 54, 55, 56, and 59.*

variation shown in the top curve is due to uncontrolled variation during the experiment.

Simple calculations indicate that the bulk of the catalatic activity can occur in the few moments while the concentration of the intermediate compound is constant — in fig. 3 e $= 1.36 \times 10^{-6}$ μM catalase, $x_0 = 15 \times 10^{-6}$ M H_2O_2. Since so little H_2O_2 is present the turnover of substrate will be governed entirely by the rate of combination of enzyme and substrate in the catalatic activity. Using the value just determined (3×10^7), the peroxide concentration can fall to a value roughly equal to the enzyme concentration in

$$\frac{2.3 \log \frac{15}{1.36}}{3 \times 10^7 \times 1.36 \times 10^{-6}} = 0.06 \text{ s}$$

— approximately the time interval that the concentration of the intermediate compound of fig. 3 remains constant. Therefore, this compound *does not begin to decompose until the bulk of the catalatic activity is complete* and the hydrogen peroxide concentration has fallen to a very low value (see »Controls»).

If, however, the substrate concentration approaches the saturation value for the catalatic activity (0.025 M^2), the »saturated» interval for this intermediate compound may be expected to increase and this is shown in fig. 8 where the hydrogen peroxide concentration is 4 mM. It is interesting that

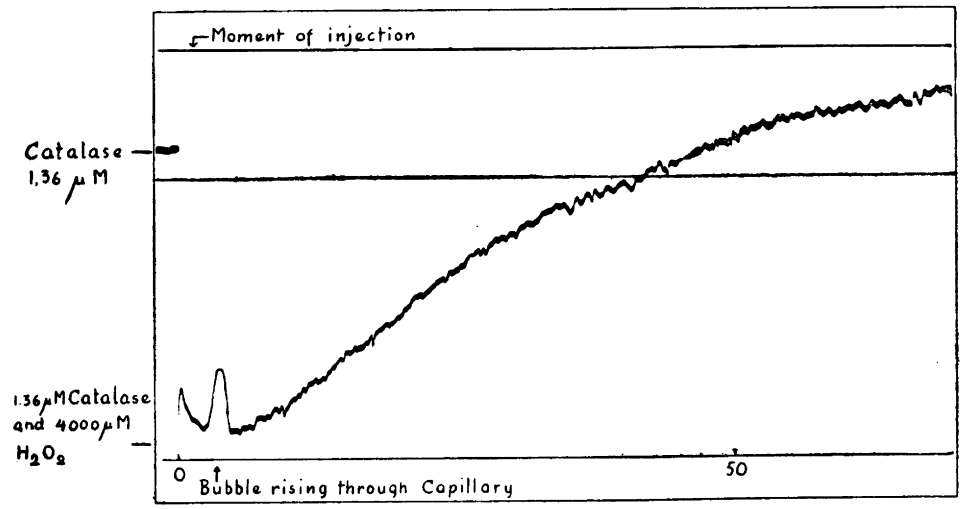

Fig. 8. The kinetics of the intermediate compound in the presence of a large initial excess (4 mM) of hydrogen peroxide. The conditions are otherwise as in fig. 3 except that the solutions were bubbled with H_2 to increase the solubility of O_2.

such a large increase of initial substrate concentration has such a small effect upon the kinetics of the intermediate compound.

Since catalase is approximately one-seventh saturated at this substrate concentration, a careful search of the spectrum was made to determine whether an addition intermediate compound could be found which had a life-time of a few hundredths of a second. So far no evidence has been obtained. It should be pointed out that these experiments are very difficult to carry out successfully since the initial portions of the curves are usually obliterated by oxygen bubbles and an inert gas was used to minimize this effect. An example of this appears in fig. 8.

THE EQUILIBRIUM CONSTANT FOR THE INTERMEDIATE COMPOUND

The relation between initial hydrogen peroxide concentration and density change is shown in fig. 9 (left), and in fig. 9 (right) the data are plotted as if a simple equilibrium existed (two opposing reactions) and on the assumption that only one of the four catalase hematins is involved in the intermediate compound. The latter assumption was required by the former since concentrations of the intermediate compound in excess of the initial peroxide concentration were obtained on the basis of more than one hematin participating in the reaction.

The value found by this method (1.6×10^{-6} M) greatly exceeds the ratio

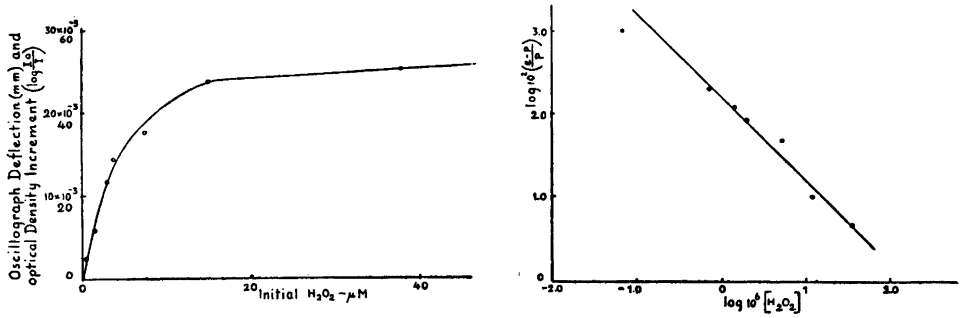

Fig. 9. The equilibrium of catalase and hydrogen peroxide. The left figure shows the variation of oscillograph deflection with initial hydrogen peroxide concentration and the right figure shows the data plotted assuming a simple equilibrium obtained and that the compound consists of one molecule of peroxide per catalase molecule. e = initial catalase, x = free H_2O_2, p = the concentration of intermediate compound.

Expt. 51 a, 3.4 μM horse blood catalase, λ = 410, s = 10, t = 25° C, pH = 6.7, 0.01 M phosphate, $t_{1/2\ off} = 14$ *s,* $k_3 = 0.05\ S^{-1}$.

of the rates of decomposition (fig. 6) and formation (table 2) of the intermediate compound (1×10^{-9} M). Therefore the equilibrium may be assumed to be composed of at least three reactions. The third is due to the high catalatic activity during the initial phases of the kinetics of the intermediate compound. (A high catalatic activity during the decay of the intermediate compound has already been shown to be unlikely because of the relation between $t_{1/2\,\mathrm{off}}$ and hydrogen peroxide concentration (fig. 7)). A first-order constant (k_7) for the catalatic activity is obtained, $K_c = \frac{k_3 + k_7}{k_1}$, $k_7 = 1.6 \times 3 \times 10^{-6+7} - 0.02 = 48\ \mathrm{s}^{-1}$, a value quite large compared with k_3. Since the catalatic activity is likely to occur at substrate concentrations slightly in excess of that of the enzyme (again since the intermediate compound decays slowly), a second-order constant is calculated; $\frac{48}{4 \times 10^{-6}} = 1.2 \times 10^7\ L \times M^{-1} \times s^{-1}$.

This value has a striking resemblance to the rate of combination of substrate with enzyme (3×10^7) and immediately suggests that the catalatic activity may be due to consecutive reactions of enzyme and substrate. Since a relatively stable compound (the intermediate compound) is seen by kinetic data (fig. 3) to exist throughout the catalatic activity, it is concluded that the catalatic reaction is due to a combination of more substrate molecules with the intermediate compound to yield a very unstable compound.

This sort of mechanism has been repeatedly proposed for hemin and for catalase activity[16, 17, 18]. In neither of these cases was any mechanism indicated whereby a peroxidatic activity and a very *high* catalatic activity could be obtained. In fact the experimental data for hemin[16], table 2) show just the opposite, catalatic/peroxidatic = 1×10^{-3}. The figure is about 4×10^4 for catalase, a net discrepancy of 4×10^7.

Unfortunately, the possibility of consecutive reactions vitiates the calculation of the composition of the intermediate compound on the basis of a simple equilibrium. The saturation value for the intermediate compound will vary with the ratio of k_1/k_7 and unless this ratio is known the calculation of the equilibrium constant cannot be made with accuracy. The calculation for k_7 given above must also be in error for the same reason.

To summarize, the catalatic activity during the initial phases of the kinetics of the intermediate compound results in the decomposition of an appreciable portion of the initial hydrogen peroxide in a reaction which has not yet been studied. The calculation of the equilibrium constant and the composition of the intermediate compound require such data. Until the composition of the intermediate compound is definitely known, the mechanism for the catalatic and peroxidatic action cannot be given with finality.

CONTROLS

A large number of control experiments were carried out in addition to those contributing to our understanding of the enzyme mechanism. Perhaps the most valid control is that of the next section which shows that this compound has sufficient activity to explain Keilins »coupled oxidation» of alcohols by catalase and hydrogen peroxide. However, an enumeration follows:

1. Does the apparatus show artifacts on mixing catalase and buffer? None in excess of 2 % of the maximum deflection and those are due to a weak mechanical mounting of the syringe unit. There is negligible drift of the baseline.

2. Is this a photochemical reaction excited by light at the peak of the Soret band? Certainly not under the conditions of 1. In the presence of hydrogen peroxide there is no difference irradiating the capillary first through a monochromator or directly from the tungsten lamp, followed by a filter (see fig. 5 and »Discussion»).

3. Is all the catalase liberated at the conclusion of the cycle? This is tested by immediately following a kinetic curve, for example that of fig. 3, by a mixture of the same catalase solution and buffer; the hydrogen peroxide is omitted. The baseline thus obtained corresponds with that obtained at the end of the cycle within the experimental error (5 %).

4. Was these an impurity in the H_2O_2 or buffer solutions? For the buffer solutions see 1. For the H_2O_2, the affinity data must indicate that the H_2O_2, if impure, is nearly completely impure and titres satisfactory with $KMnO_4$.

5. Were there impurities in the catalase preparations? In addition to the data of table 1, a particular catalase preparation was followed from start to finish and in all stages after the first, the compound was satisfactorily demonstrated. Also the same reasoning as 4 applies.

6. Since the compound is stable for a few moments can the measurement be made in the ordinary apparatus? Rapid manipulation of the Beckman spectrophotometer gives a measurable spectral shift at 405 mμ on addition of H_2O_2 to alcohol free catalase. The average of three experiments with liver catalase gave $(\Delta\varepsilon_1)\lambda = 405 = 38$; in good agreement with the data of table 1. This procedure is, however, not recommended for accurate determinations.

7. Was H_2O_2 present during the decay of the intermediate compound? A number of attempts were made to demonstrate the presence of free hydrogen peroxide during the decompostion of the intermediate compound. The following methods were tried:

1. Ceric sulfate.
2. Peroxidase and ascorbic acid.
3. Azide catalase.

In each case the peroxide-sensitive indicator was mixed with catalase (2 μM) and a large excess of H_2O_2 ($\approx$ 0.5 mM) within 15 seconds after mixing the latter two components. The sensitivity of the last two methods was surely adequate to detect 1 μM H_2O_2. The last tests will be explained in detail.

Catalase (2 μM) and H_2O_2 (0.4 mM) were mixed in a test tube and rapidly transferred to one syringe of the rapid reaction apparatus (10 s). This mixture was immediately mixed with 40 μM azide in the capillary and the azide-catalase H_2O_2 compound was sought at 430 mμ. The procedure was similar in 2 but azide was replaced by peroxidase and ascorbic acid.

In no case was there any evidence of H_2O_2 in excess of the experimental error. The conclusion is that the catalatic activity removed nearly all the H_2O_2 in a short time while the high affinity of the intermediate compound held the remainder from the indicators. In the case of 3 it is believed that a small amount of H_2O_2 could be demonstrated provided the test were carried out more rapidly.

THE PEROXIDATIC PROPERTIES OF THE INTERMEDIATE COMPOUND

Since this compound appeared to have may properties in common with peroxidase-H_2O_2, a number of acceptors were added to determine their effect upon the stability of the compound.

Ascorbic acid

The addition of a millimole of ascorbic acid to the intermediate compound formed in the usual way caused a definite acceleration of its decomposition. Since catalase has a very high affinity for hydrogen peroxide, it was possible to form the intermediate compound directly from the hydrogen peroxide present in ascorbic acid solution. The data of fig. 10 show the increase in the amount of intermediate compound and the increase in the rate of its decomposition as a function of initial ascorbic acid concentration. The acceleration of the rate is more than two fold for 80 μM ascorbic acid and a velocity constant of 360 $L \times M^{-1} \times s^{-1}$ is obtained by deducting the »blank» rate. This is quite low compared with the activity of horse-radish peroxidase which has a constant of 1.8×10^5 $L \times M^{-1} \times s^{-1}$. It appears that the influence of the protein component of catalase has resulted in a different acceptor specifi-

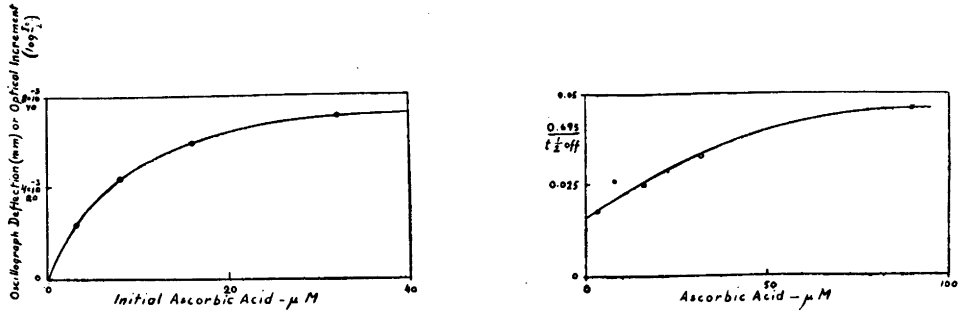

Fig. 10. The formation of the intermediate compound in the presence of ascorbic acid (left). The acceleration of its decomposition by ascorbic acid (right) ($k_4 = 360$ $L \times M^{-1} \times s^{-1}$). Expt. 55, 1.36 μM horse blood catalase, $\lambda = 405$, $s = 30$, $t = 23°$ C, pH = 6.7, 0.01 M phosphate.

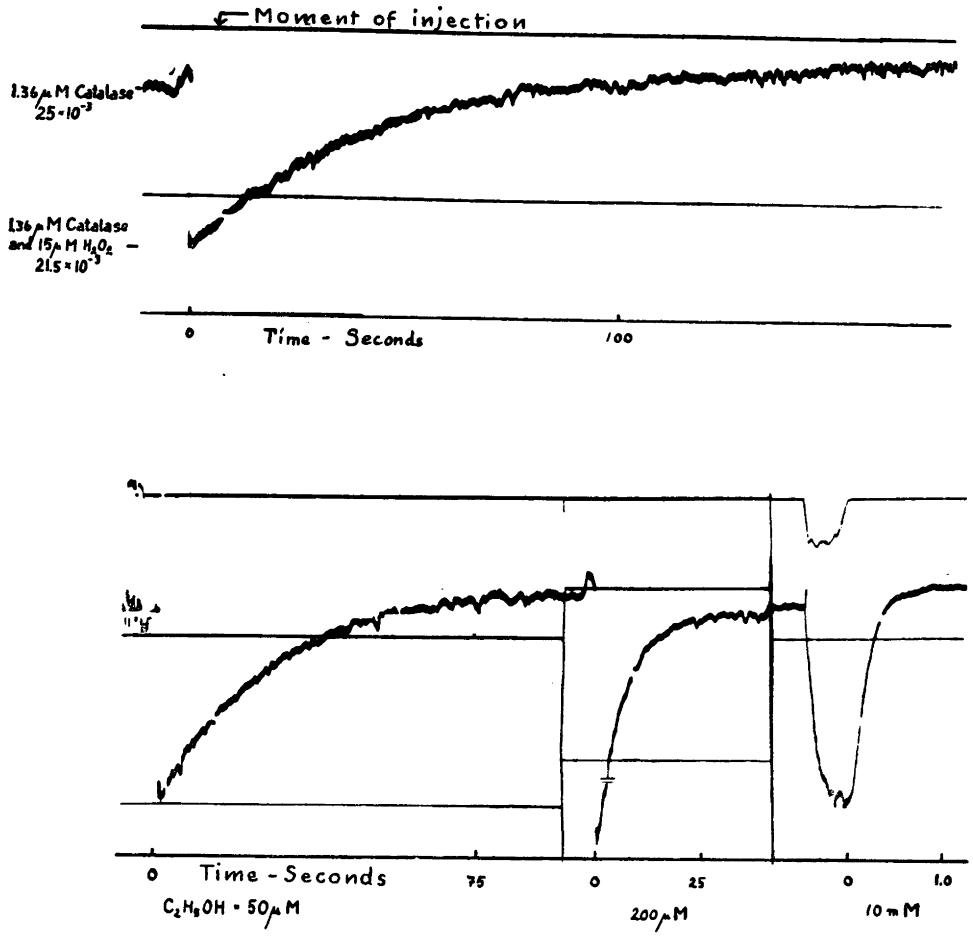

Fig. 11. The kinetics of the intermediate compound in varous alcohol concentrations. The alcohol concentration is zero in the top record.

city and that other types of acceptors must be employed in order to obtain optimal acitivity, for example, the alcohols used in Keilins »coupled oxidations»[6].

The effect of ethanol

The rather dramatic effect of ethanol upon the stability of the intermediate compound is shown in the series of kinetic curves of fig. 11. The half-life of the compound is decreased from 30 to 0.1 s by 10 *mM* ethanol. Fig. 12 shows that the effect of ethanol is to increase in a regular fashion the first-order

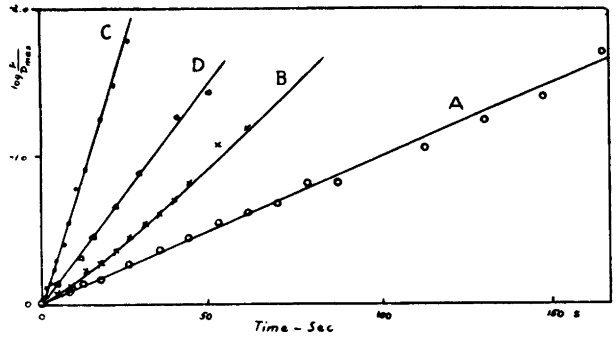

Fig. 12. The order of the reaction. The data of fig. 11 are plotted according to a first order equation. Note that abscissa is divided by 180 for the 10 mM curve (D). A = 0, B = 50, C = 200, D = 10,000 μM ethanol.
Expt. 59, 1.36 μM horse blood catalase, 12 μM H_2O_2, λ = 405, S = 30, t = 24° C, pH = 6.7, 0.01 M phosphate.

velocity constant for the decomposition. This suggests that there is a second-order reaction of ethanol and the intermediate compound and the data of fig. 13 verify this. Since the rate of decomposition is not zero with zero alcohol concentration, the original data appear as the two curves (circles and crosses) approaching a straight line asymptotically. Subtraction of the »blank» rate

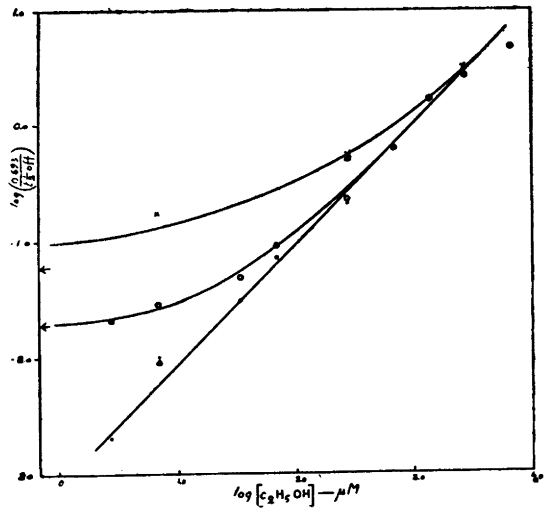

Fig. 13. The second-order velocity constant k_4 = 1000 L × M^{-1} × s^{-1} for the reaction of the intermediate compound and ethanol. The curves are uncorrected for the blank reaction.
o, · Expt. 59, 1.36 μM horse blood catalase, 15 μM H_2O_2.
×, Δ Expt. 62, 1.94 μM horse liver catalase, 6 μM H_2O_2.
λ = 405, S = 30, t = 20° C, pH = 6.7, 0.01 M phosphate.

from the original data gives the remainder of the points on the straight line. At the highest concentration of alcohol there is some evidence of a »saturation» effect and experiments with still higher alcohol concentrations verify this; 0.085 *M* ethanol gives a constant of only 100 L × M^{-1} × s^{-1}. The breakdown constant for such an alcohol-peroxide-catalase complex can be estimated from the value $k_4 = 100$ above since $100 \times 0.085 = 8.5$ s^{-1} or roughly 10 s^{-1}. assuming that a further increase of alcohol brings about a slight further increase of rate. Thus a »Michaelis Constant» would be roughly 10^{-2} *M*.

This effect is very similar to that of ascorbic acid upon the intermediate compound of peroxidase and hydrogen peroxide and may be termed a peroxidatic activity of catalase. Apparently the extreme linearity of the effect of alcohol concentration is only limited by the rate of breakdown of a catalase-peroxide-alcohol complex as is also the case in the reaction of peroxidase and ascorbic acid or leucomalachite green [7]. The peroxidatic activity of catalase for ethanol is, however, less than that of peroxidase for ascorbic acid, 1×10^3 compared with 1.8×10^5, although the maximum turnover ($k_5 \approx 10$ s^{-1}) does not greatly differ.

As in the case of peroxidase and ascorbic acid, the rate of decomposition is not accelerated by mixing the alcohol with the enzyme before mixing with the hydrogen peroxide compared with the results obtained on mixing the enzyme with substrate and acceptor sumultaneously (at least up to 4 *mM* ethanol).

It is unfortunately much more difficult to correlate the kinetics of the intermediate compound with the turnover of ethanol than in the case of peroxidase where dyes of intense absorption are produced (malachite green, etc.). Also in these studies the maximum amount of ethanol consumed would be related to the *enzyme concentration* and not to the peroxide concentration since the excess peroxide is removed by the catalatic pathway. However, Keilins' [6] experiments were all carried out with a continuous supply of H_2O_2 from either an enzyme system or a mechanical device, and the production of aldehyde from alcohol was demonstrated by the Schiff test. It is possible that the decomposition of the intermediate compound could cause the oxidation of an equivalent amount of alcohol and its activity may be compared with that found in Keilins' manometric tests.

From Keilins' fig. 1, the rate of oxygen uptake during the initial phase of the reaction is 145 μl in 20 min. or 5.4×10^{-3} μM/s in terms of O_2, hydrogen peroxide, ethanol, or acetaldehyde. The amount of catalase present is 8.7×10^{-3} μM and hence the turnover is 0.7 s^{-1}.

In these experiments, k_4 has fallen to 100 L × M^{-1} × s^{-1} at this high alcohol concentration and the rate of breakdown of the intermediate compound is 100 × 0.07 *M*

$= 7\ s^{-1}$, a turnover ten times as great as in Keilins' experiments if the assumption is made that the decomposition of the intermediate compound in the presence of alcohol gives an equivalent amount of aldehyde.

In this case it is clear that *the intermediate compound can easily be responsible for the »coupled oxidation» in Keilins' experiments.*

The smaller turnover is probably due to the partial saturation of catalase with H_2O_2 furnished by the enzyme system. This will also explain the data of Keilins' table 4 where the first ten-fold reduction of the enzyme concentration caused less effect than subsequent ten-fold reductions. As the catalase concentration is decreased, first the saturation will increase without much loss of turnover. However, when catalase is saturated, the excess of hydrogen peroxide will be decomposed catalatically resulting in the decrease of oxygen uptake shown in Keilins' table 4. The ratio of catalatic to peroxidatic activity is a function of the relative concentrations of catalase and hydrogen peroxide.

The effect of alcohol upon the intermediate compound may then be formulated in general terms. The bulk of the data indicate that n = 1.

$$\underbrace{\mathrm{Cat} + \mathrm{n(H_2O_2)}}_{\substack{\text{Very rapid combination} \\ k_1 \approx 3 \times 10^7\ \mathrm{L} \times \mathrm{M^{-1}} \times \mathrm{s^{-1}}}} \xrightarrow{k_1} \underbrace{\mathrm{Cat(H_2O_2)_n} + \mathrm{nC_2H_5OH}}_{\substack{\text{Rapid second order reaction} \\ k_4 = 100\ \mathrm{L} \times \mathrm{M^{-1}} \times \mathrm{s^{-1}}}} \xrightarrow{k_4} \underbrace{\mathrm{Cat(H_2O_2)_n(C_2H_5OH)_n}}_{\substack{\text{Relatively unstable compound} \\ k_5 \text{ estimated } 10\ \mathrm{s^{-1}}}} \xrightarrow{k_5} \mathrm{Cat} + 2\mathrm{nH_2O} + \mathrm{nCH_3CHO}$$

Spectrum

The spectrum of the intermediate compound in the presence of alcohol was measured for these reasons. 1) The measurements can be carried out in more dilute solutions since the »cycle» is more rapid and slow fluctuations of the light intensity have less effect. 2) It is of interest to determine whether an intermediate compound of catalase, peroxide, and alcohol can be detected.

Using a 1 *mM* ethanol concentration, the spectrum of fig. 14 was obtained by the same calculations as used for fig. 4. The shape of the curve and the values of extinction are equal to those of fig. 4 to within the experimental error. Therefore neither a change of enzyme concentration (or source) nor the presence of alcohol alter the spectrum appreciably. Thus there is only kinetic evidence for the catalase-hydrogen peroxide-alcoholcomplex.

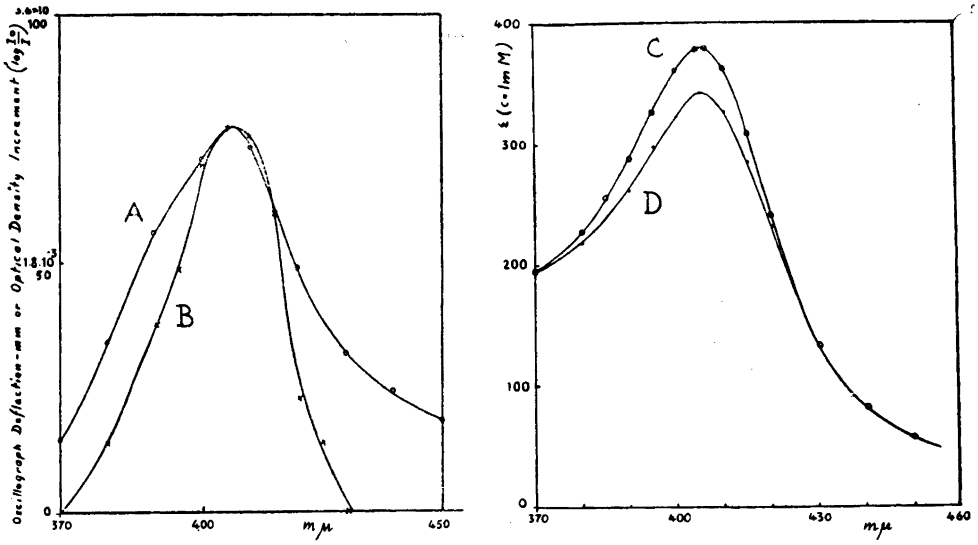

Fig. 14. The spectrum of the intermediate compound in the presence of ethanol. Other features as in fig. 4. A = catalase compared with water, B = catalase compared with the intermediate compound, C = catalase, D = catalase-hydrogen peroxide spectrum.
Expt. 66, × = 1.63 M horse kidney catalase, 12 M H_2O_2 1 mM C_2H_5OH, 0 = 0.16 μM horse kidney catalase, S = 30, t = 23° C, pH = 6.7, 0.01 M phosphate.

The equilibrium constant in the presence of alcohol

Fig. 15 shows the increase of the concentration of the intermediate compound with increasing hydrogen-peroxide concentration. Similar data are plotted in fig. 16 using the same assumptions as fig. 9 and with approximately

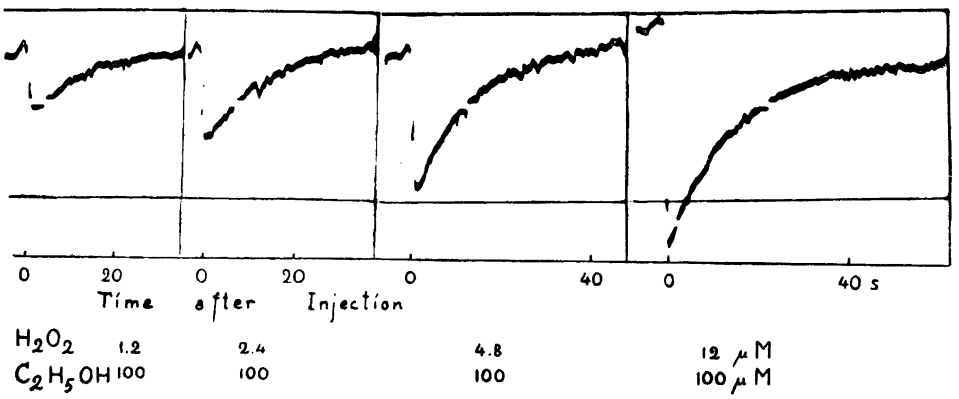

Fig. 15. The effect of hydrogen peroxide upon the kinetics of the intermediate compound in the presence of 100 μM ethanol.

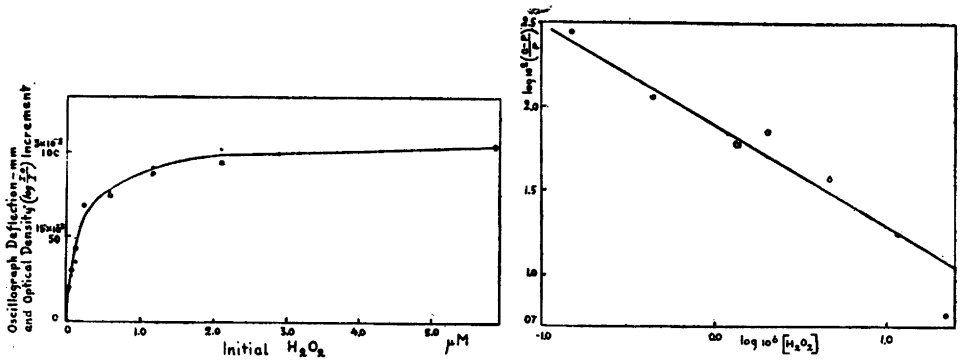

Fig. 16. The equilibrium of catalase and hydrogen peroxide in the presence of ethanol. assumptions were the same as in fig. 9. $K_M = 0.8\ 10^{-6}\ M$, $a = 0.58$, *Expt. 66 d, 1.6 μM horse kidney catalase, 1 mM ethanol,* $\lambda = 405$, $S = 50$, $t = 26°\ C$, $pH = 6.7$, *0.01 M phosphate.* $t_{1/2\ off} = 0.7\ s$, $k_4 = 1000\ L \times M^{-1} \times s^{-1}$

the same result except that a line of slope = 0.58 was drawn. No explanation of this difference is proposed.

The increase of alcohol concentration should lead to an increase in the amount of hydrogen peroxide required to saturate the intermediate compound.

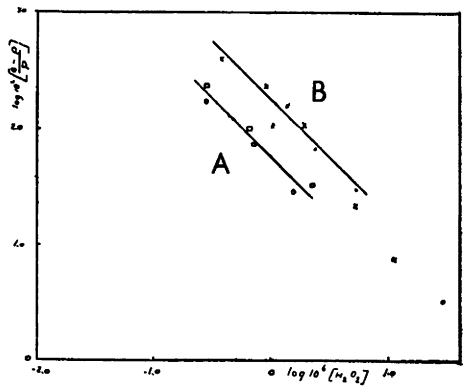

Fig. 17. The effect of ethanol upon the equilibrium constant. The assumptions were the same as in fig. 9.

For curve A, $K_M = 0.56\ 10^{-6}\ M$, $a = 1$,
For curve B, $K_M = 1.7\ 10^{-6}\ M$, $a = 1$.
Expts. 60 a + 61, 1.1 M horse blood catalase,
$\square = 0$, $O = 100$, $\times = 400$, $\cdot = 4000\ \mu M\ C_2H_5OH$,
$\lambda = 405$, $S = 30$, $t = 25°\ C$, $pH = 6.7$, *0.01 M phosphate.*

This effect is qualitatively illustrated by fig. 17 were there is a shift of the »constant» from 0.56 to 1.7 $\times$ 10^{-6} M from the lower to the higher concentrations. As in the case of the fig. 9, the effect of the catalatic disappearance of H_2O_2 must not be forgotten and not only is the equilibrium constant much larger than the ratio of the measured rates of decomposition and formation of the intermediate compound but it must be also relatively insensitive to a change in the rate of decomposition as indeed it is. In fact a rate constant for the catalatic activity can be now estimated for both values of the equilibrium constant: $\frac{0.03 + k_7}{3 \times 10^7} = 0.56 \times 10^{-6}$ and $\frac{4.0 + k_7}{3 \times 10^7} = 1.7 \times 10^{-6}$ giving 18 and 49 s^{-1} respectively or in second order units, between 2 and 5 $\times$ 10^7. Apparently an accurate value for the catalatic activity cannot be found on the basis of such simple assumptions.

The effect of excess hydrogen peroxide

As already illustrated in fig. 7 for the absence of alcohol, the rate of decomposition of the intermediate is not greatly affected by excess hydrogen peroxide, presumably because the catalatic activity rapidly removes this excess. This presumption is strongly supported by the data of fig. 18 and, since the decay times are shorter, the data are more reliable than those of fig. 7.

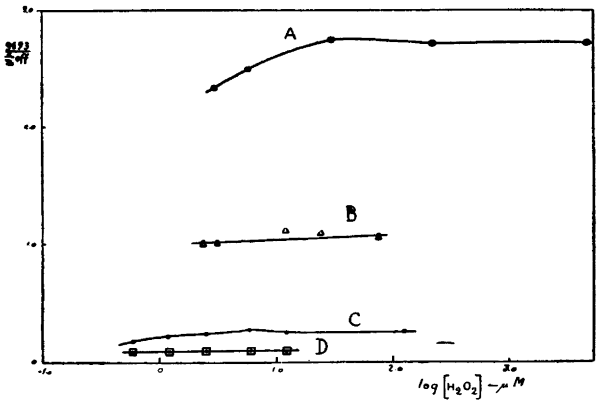

Fig. 18. The effect of initial hydrogen peroxide concentration upon the rate of decomposition of the intermediate compound in the presence of varying amounts of alcohol.

Expts. 60 a + b, 61, and 66.
A = 4000 = B 1000, C = 400, D = 100 μM C_2H_5OH.

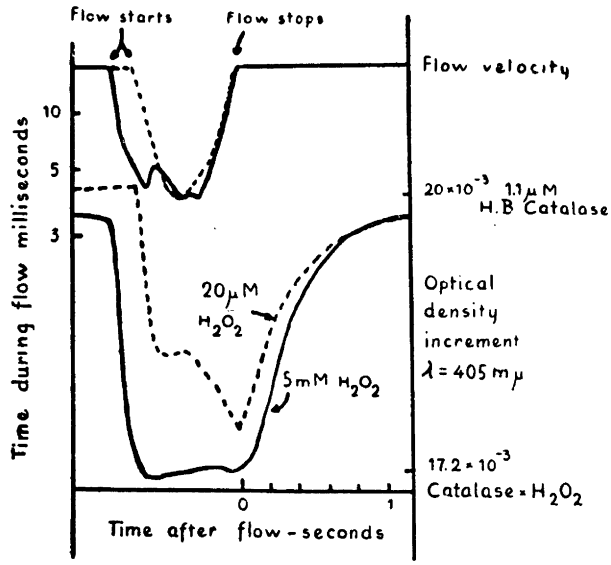

Fig. 19. The competition between hydrogen peroxide and ethanol for the intermediate compound. Excess initial hydrogen peroxide causes a delay of about 0.1 s. Expt. 67b. Other conditions as in fig. 8 except no H_2 was used.

The competition between ethanol and hydrogen peroxide for the intermediate compound

In the absence of alcohol, fig. 8 showed a considerable delay in the decomposition of the intermediate compound in the presence of excess hydrogen peroxide. When alcohol is present it and the last traces of hydrogen peroxide would be expected to compete for the intermediate compound and thereby to cause a considerable reduction of the delay. This is demonstrated by fig. 19 where the tracings of two kinetic curves with 20 μM and 5 mM H_2O_2 are shown. The delay in strong peroxide and alcohol is about 0.1 s. This again indicates that the intermediate compound does not begin to decompose until the catalatic activity is complete.

The effect of a variation of enzyme concentration

Since the rapid kinetics can be recorded more reliably, an illustration of the effect of a five-fold decrease of enzyme concentration is shown in fig. 20. The activity of the enzyme is the same and the greater instability of the apparatus in the more dilute solution causes only minor differences between the two curves.

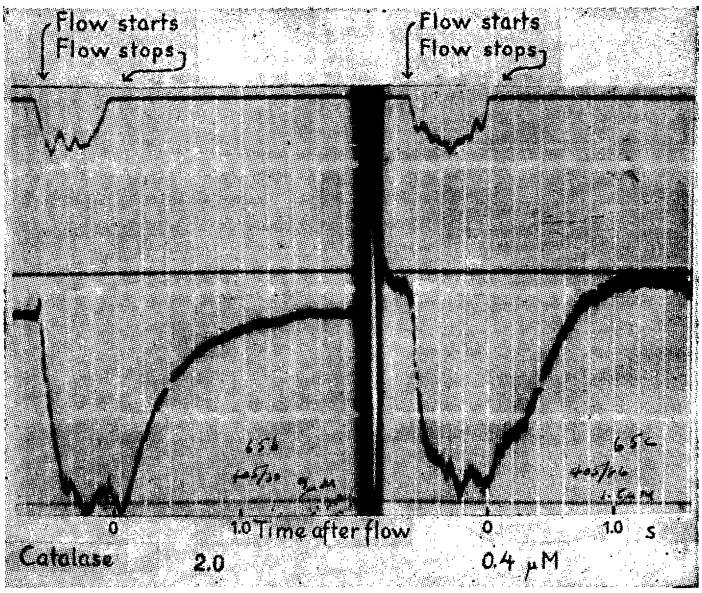

Fig. 20. The effect of a five-fold variation of enzyme concentration upon the kinetics of the intermediate compound in the presence of 4 mM C_2H_5OH and 12 μM H_2O_2.

The effect of other alcohols

Methanol. Fig. 21 shows that the variation of the corrected rate is linear over about the same range as with ethanol and that a similar saturation effect occurs. Within the experimental error the velocity constants are identical although the volatility of methanol might cause the values to be somewhat low.

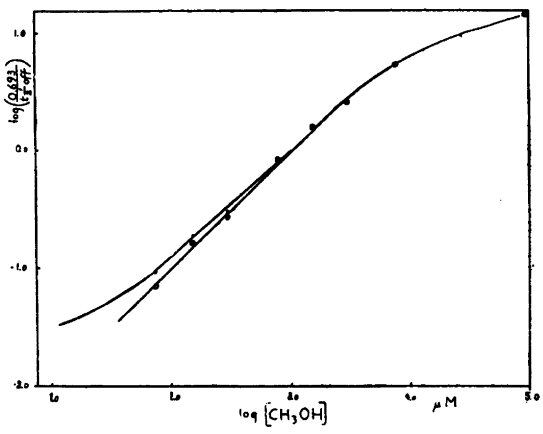

Fig. 21. The second-order velocity constant $k_4 = 100\ L \times M^{-1} \times s^{-1}$ for the reaction of the intermediate compound with methanol. The left-hand curve is uncorrected for the blank rate.
Expt. 70, 1.85 μM horse blood catalase, 30 μM H_2O_2 λ = 405, S = 30, t = 22° C, pH = 6.7, 0.01 M phosphate.

Agner* has recently determined the kinetics of the disappearance of methanol from the blood of rabbits [19]. The data of fig. 3 fit a first order equation having a rate constant of 1.8×10^{-5} s^{-1}. It has been shown that methanol is oxidized to formaldehyde in the liver [20]. The liver catalase concentration calculated on the basis of the liquid phase (since the alcohol is uniformly distributed in this phase) is 0.17 μM.

For those experiments, the saturation of catalase is assumed to be constant and the alcohol to disappear according to the following equation:

$$\underset{a}{CH_3OH} + \underset{p}{Cat \cdot H_2O_2} \xrightarrow[k_4]{} HCHO + 2H_2O + Cat$$

$$\frac{da}{dt} = k_4 ap \text{ (at } t = 0,\ a = a_o); \log \frac{a_o}{a} = k_4\ p\ t.$$

Since Agner's figure is k_4p, p was assumed equal to e,

$$\log \frac{a_o}{a} = k_4\ e\ t$$

$$k_4 e = 1.8 \times 10^{-5}\ s^{-1}$$

$$k_4 = \frac{1.8 \times 10^{-5}}{0.17 \times 10^{-6}} = 100\ L \times M^{-1} \times s^{-1}$$

But in these experiments with the intermediate compound the kinetics of p, and not a, were measured; $\log \frac{e}{p} = k_4\ a_o t$, and k_4 is found to be 1000 L $\times$ M^{-1} $\times$ s^{-1} for low methanol concentrations and roughly 500 L $\times$ M^{-1} $\times$ s^{-1} over the range of alcohol concentrations used in Agner's experiments five times that required by his data.

It is seen that more than enough catalase is present in the rabbit liver to account for Agner's kinetic data. Furthermore, if it is assumed that all the liver catalase is equally exposed to methanol in this reaction, the saturation of the enzyme would appear to be about 20 % and the peroxide concentration (supplied by other enzyme systems) in the neighborhood of 1 μM in this liver.

Therefore the knowledge of the kinetics of methanol uptake of an organ, its catalase content, and the velocity constant for the intermediate compound and methanol (1000 L $\times$ M^{-1} $\times$ s^{-1}) permits a determination of the hydrogen peroxide turnover in that organ, and very small concentrations of hydrogen peroxide may readily be determined. It is suggested that this technique may be a useful tool in physiology.

It is also of interest that the concentration of H_2O_2 found in rabbit liver is so low — one wonders whether catalase ever acts catalatically *in vivo*. On the other hand it would be difficult to imagine a substance that could be better suited to permitting a small concentration of H_2O_2 to exist for metabolic processes and also for rapidly decomposing a concentration of H_2O_2 in *excess* of the amount of catalase. Certainly the H_2O_2 and catalase concentrations found in this experiment are too nearly equal to permit a clear cut decision.

There is still no evidence of the relation between alcohol dehydrogenase and catalase and, although these data clearly indicate that catalase is present in rabbit liver in sufficient concentration to account for Agner's data, no proof is yet afforded that catalase and

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* These calculations were made in collaboration with Docent Agner.

not alcohol dehydrogenase actually carried out the oxidation. Also the inhibition of methanol oxidation by ethanol as observed *in vivo*[19] and *in vitro* with alcohol dehydrogenase[21] is unlikely in the case of catalase unless ethanol inhibits those enzyme systems which, *in vivo*, supply catalase with hydrogen peroxide.

A comparison of five alcohols. Fig. 22 shows the relative rates of combination of various alcohols with the intermediate compound. While ethyl and methyl alcohol have quite similar rates, *n*-propanol and *n*-butanol are respectively only 1/60 and 1/500 as effective. The rate with the highest concentration of iso-amyl alcohol is so nearly equal to the blank rate that the difference is subject to considerable error. The data also indicate that saturation may occur at a lower concentration with the higher alcohols. It is quite possible that steric factors are responsible for the slower rates of combination of the higher alcohols.

These data may be compared with Keilins'[6] figs. 3 and 4 with the following reservations.

1. Since the catalase is not completely saturated with H_2O_2, Keilins' system may be non-linear for small changes of the rate of combination of alcohol and intermediate compound.

2. It is essentially the rate of breakdown of the alcohol-peroxide-catalase compound that is measured in Keilins' experiments since his alcohol concentrations approach the saturation values.

3. Apparently his results vary with the enzyme system and/or the turnover number. In fig. 3 the effect of ethyl to propyl was equivalent to a 10 fold reduction of catalase concentration (table 4) while in fig. 4 the effect is equivalent to in excess 100 fold reduction of catalase (table 4). A greater sensitivity with a higher turnover would be expected.

4. The small but consistent difference between ethanol and methanol shown in Keilins' data may be attributed to 2.

Although there are differences in the conditions under which the relative activities were measured by the two methods, there are certainly no differences concerning the decrease of activity with the higher alcohols.

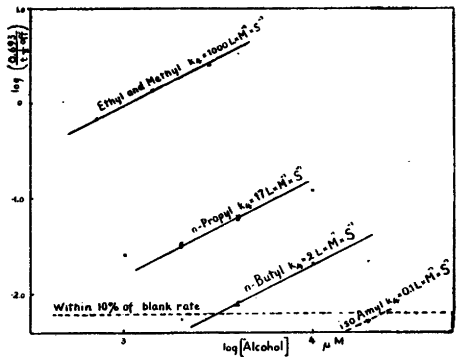

Fig. 22. A comparison of the reaction velocity of the intermediate compund with five different alcohols. Expt. 71, 1.85 μM horse blood catalase, 20 μM H_2O_2 λ = 405, S = 30, t = 23° C, pH = 6.7, 0.01 M phosphate.

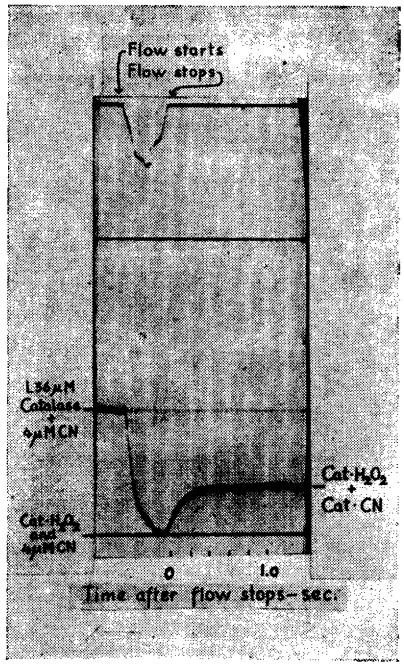

Fig. 23. The kinetics of the intermediate compound in the presence of cyanide — initial phase only.

The effect of inhibitors

Cyanide. The competition between hydrogen peroxide and cyanide is shown in fig. 23. At this wavelength (420 mμ) the density change due to the catalase-cyanide compound (+) has the opposite sign to that of the intermediate compound (—). The abrupt drop in density at the moment of injection is due of to the replacement of catalase-cyanide compound remaining in the capillary from the preceeding injection by a mixture of the intermediate compound, catalase, and cyanide. The cyanide then rapidly combines with a portion of the catalase as indicated by the increase of density after the flow stops. The intermediate compounds is momentarily stable but decays as shown in the first of the series of curves of fig. 24 which are recorded on a much slower time scale.

This series of curves shows the regular increase of the concentration of the cyanide compound and a decrease of the concentration of the intermediate compound as the cyanide concentration is increased. In fact in the last two records very little intermediate is formed.

Azide. The effect of azide is not so straightforward because: 1) more than one azide-catalase-peroxide compound can form and 2) the azide-catalase-

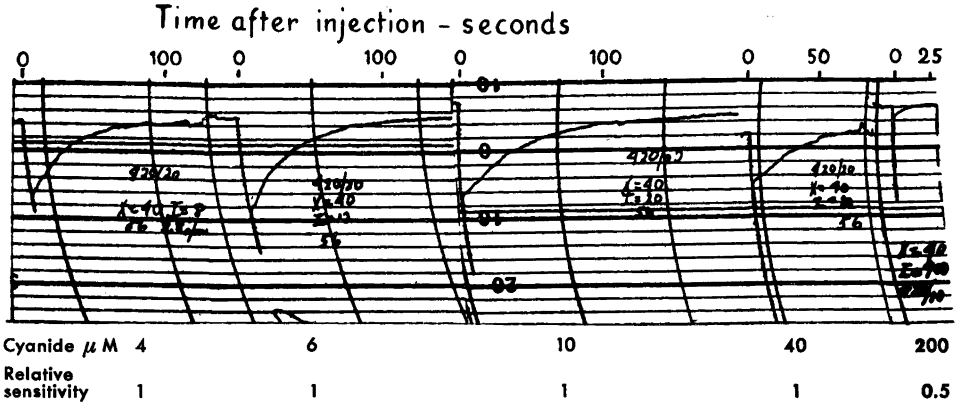

Fig. 24. The kinetics of the intermediate compound in the presence of varying amounts of cyanide. Note that the sensitivity was reduced in the last figure.

peroxide compound itself decomposes in a few minutes. Fig. 25 shows at 410 mμ that the rapid formation of the intermediate compound is followed by the slow formation of the azide-catalase-peroxide compound when dilute azide is used (10 μM). With more azide (100 μM) the distinctive spectrum and kinetics of this compound are shown in fig. 26 on a slower time scale. In these records, however, the initial phase appears to consist of the primary formation of the intermediate compound followed by the formation of at least two different types of azide-catalase-peroxide compounds during the course of its decomposition. A wide variation of experimental conditions does not appear to alter the rate of formation of the azide-catalase-peroxide compound and, therefore, it is concluded that *the extremely high affinity of this compound,* to quote Keilin[5] »Azide-catalase is certainly one of the most sensitive reagents for the detection of H_2O_2 . . . . . .», *is due entirely to the high affinity of the intermediate compound,* and not azide-catalase, *for* H_2O_2. This finding does not conflict with previous data, it merely explains them.

The nature and significance of the complex kinetics of the azide-catalase-peroxide compounds shown in fig. 26 is indeed beyond the scope of this paper. A significant factor is, however, that the life-time of this compound is quite independent of hydrogen peroxide concentration over a very wide range (in fact — as large as that of the intermediate compound). Under these experimental conditions, the same explanation may apply; the catalatic pathway first reduces the peroxide concentration to a very low value and then the azide-catalase-peroxide compound forms and goes through its complicated course of decomposition.

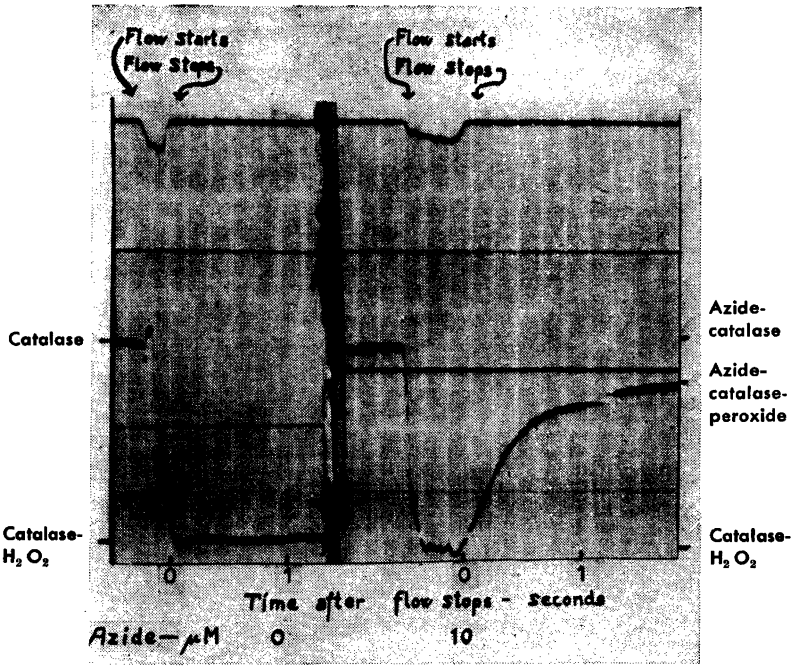

Fig. 25. The effect of 10 μM NaN_3 upon the kinetics of the intermediate compound 400 μM H_2O_2.

Since azide-catalase-peroxide decomposes at a constant rate, can this be accelerated by alcohol? Evidently the peroxidatic activity is completely inhibited by 100 *μM* azide since 1 *mM* ethanol has no effect upon the kinetics of the azide-catalase-peroxide compound.

Ethyl hydroperoxide. With strong ethyl hydroperoxide (0.1 *M*) as used by Stern [4] the intermediate is rapidly converted into the ethyl hydroperoxide compound.

Carbon Monoxide. There is no inhibition of the effect of 4 *mM* ethanol by 500 *μM* CO. Also there are no measurable spectral changes under aerobic or anaerobic concitions.

Anaerobic conditions. The rate of decomposition of the intermediate compound in the presence (4 *mM*) or absence of ethanol was not greatly affected by anaerobic conditions. (Solutions were bubbled for 20 min. with H_2 passed over heated platinized asbestos.)

The data above strong support the view that the intermediate compound of peroxide attached to hematin and that no detectable amount of a reduced

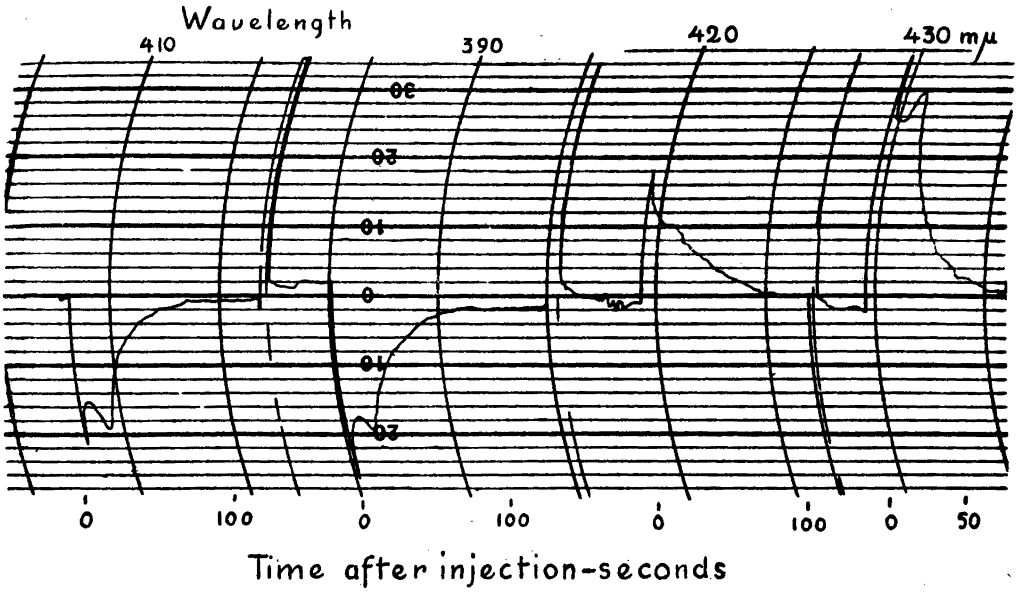

Fig. 26. The kinetics of azide-catalase-peroxide at three wavelengths — 100 μM NaN_3, 400 μM H_2O_2.

compound is formed during the kinetics of the intermediate compound. This further increases the similarity to peroxidase — H_2O_2.

SUMMARY

A complete discussion of these and other data will appear in a series of papers[22]; at present the results are only summarized as follows:

1. Catalase forms an intermediate compound in the presence of hydrogen peroxide.
2. This compound reacts with acceptors and inhibitors much in the same way as peroxidase — H_2O_2 and is believed to be a similar peroxide complex with catalase.
3. The spectrum of the intermediate compound has been measured from 380 to 430 mμ and is slightly shifted towards the visible with respect to catalase. $\varepsilon_{405\ (c\ =\ 1\ mM)} = 340$ for blood catalase. The spectrum is unlike that of cyanide-catalase or azide-catalase-peroxide.
5. The rate of formation of the complex is very high and exceeds the value required for the catalatic activity of the enzyme as well as those for peroxidase and myoglobin with hydrogen peroxide and oxygen respectively. A preliminary value for the constant is 3×10^7 L × M^{-1} × s^{-1},

6) With pure crystalline catalases the intermediate compound decomposes very slowly and with slight variability from preparation to preparation as in the case of peroxidase-hydrogen peroxide. An average value for the breakdown constant is 0.02 s^{-1}.

7. The intermediate compound has a very high affinity for hydrogen peroxide. Since the calculation of the data requires the postulation of a comprehensive enzyme mechanism, no correct figure can be given as yet. The simple equilibrium equations give $1 \times 10^{-6} M$, the kinetic data give $1 \times 10^{-9} M$ indicating a large catalatic activity during the formation of the intermediate compound ($k_3 = 30\ s^{-1}$).

8. The intermediate compound reacts slowly with ascorbic acid ($k_4 = 340\ L \times M^{-1} \times s^{-1}$), more rapidly with ethyl and methyl alcohols ($k_4 = 1000\ L \times M^{-1} \times s^{-1}$) and less rapidly with the higher alcohols, *n*-propanol ($k_4 = 17\ L \times M^{-1} \times s^{-1}$), *n*-butanol ($k_1 = 2\ L \times M^{-1} \times s^{-1}$), and very slowly with iso-amyl alcohol ($k_4 = 0.1\ L \times M^{-1} \times s^{-1}$).

9. Saturation effects with excess ethanol and methanol lead to the conclusion that a further intermediate compound is formed in these reactions as in the case of peroxidase. The velocity constant for the break-down of such a complex is 10 s^{-1}, similar to the value found for peroxidase.

10. The peroxidatic activity of the intermediate compound is more than adequate to account for Keilin's experiments on »coupled oxidations» and Agner's experiments on the kinetics of methanol disappearance in vivo.

11. Where catalase is solely responsible for methanol oxidation the velocity constants in 8 permit determinations of the hydrogen peroxide concentration or turnover in vivo by simply following the kinetics of methyl alcohol oxidation and determining the catalase content.

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