These are valid equivalents; but the important point is this—the agreed figure for the last two whaling seasons of 16,000 'blue whale units' for the contracting parties may well mean that a full catch involves the killing of many more than 16,000 animals. For example, in 1945–46 the Antarctic catch was returned[6] as 8,304·8 'blue whale units', but was, in fact, made up of 13,381 whales (3,604 blue, 9,184 fin, 238 humpback and 81 sei whales, as well as 239 sperm whales and 35 "sperm and others"). This last might include some bottlenose or *Berardius*. The catch for 1946–47 has been returned[7] as 15,230·7 'blue whale units'. This represents 8,870 blue, 12,857 fin and 2 sei whales, a total of 21,729 whales[8].

There is obviously a serious danger of losing sight of the reality of the position by continued contemplation of the 'blue whale unit' value of a catch, since the number of animals killed will always be greater than the number of units allowed. An industry of this sort cannot be regulated by reference to abstractions, when it depends entirely upon a stock of wild animals, over the lives and breeding habits of which there is no control.

In the presence of vast and unestimated numbers of animals which it is desired to exploit commercially, nothing is easier than to persuade oneself that the stock is so great that it is impossible to injure it, still less to destroy it. But it is my opinion that the stocks of blue whales are showing clear signs of reduction: (1) in the rarity or absence of the huge animals of earlier seasons; (2) in the reduced proportion in the total catch of this species; and (3) in the reduced percentage of mature females which were pregnant. It is also certain that a parallel reduction of the stock of fin whales is now in progress.

The position with regard to sperm whales is somewhat different. These animals are polygamous, and there are therefore spare bulls which can very well be utilized. The sperm whales occurring outside the warm waters of the breeding grounds are almost invariably large males, which have not been able to keep their place in the herds. There is, however, no information as to the size of this stock of spare bulls, or whether they are resident in the Antarctic. Hence, the present increased killing may simply be using up the accumulation of years, either of a resident male stock, or of an annually visitant herd. The appropriate literature is not available here; but I have the clearest recollection that in the days of the old sperm whalers, certain solitary bulls were known to haunt particular areas of the sea, and continued to do so for years. Such whales, like the authentic 'Moby Dick' and 'Paita Tom', attacked boats at sight.

Finally, it may not be out of place to recall to memory the fate of the North American bison and the passenger pigeon. The former was brought to the verge of extinction and the latter completely exterminated by man for commercial purposes, in spite of the almost incredible numbers in which they both existed a little more than a century ago.

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# THE ENZYME-SUBSTRATE COMPOUNDS OF CATALASE AND PEROXIDES

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CATALASE is a hæmatin enzyme found in relatively large concentrations in erythrocytes, liver, kidney, etc. Its extremely efficient catalysis of the destruction of hydrogen peroxide and its ability to catalyse the oxidation of alcohols by hydrogen peroxide have been recognized for some time, but very little direct evidence for the mechanisms of these reactions in terms of the theory of enzyme-substrate compounds has hitherto been obtained.

Using a micro form of the method of Hartridge and Roughton[1] and Millikan[2] for rapid spectrophotometric studies[3] of the reaction kinetics and equilibria of the unstable compounds of catalase with hydrogen peroxide and methyl or ethyl hydrogen peroxide, the catalytically active enzyme-substrate compounds of catalase with these substances have been discovered. In addition, corresponding inactive forms of the compounds of catalase with hydrogen peroxide and methyl hydrogen peroxide have been revealed. The catalase–ethyl hydrogen peroxide complex which had previously been studied by Stern[4] is shown to be one of these inactive enzyme-substrate compounds. The three active enzyme-substrate complexes share a common property of oxidizing substances such as alcohols, and in this case catalase functions as a peroxidase. But the active catalase–hydrogen peroxide complex has the unique property of acting 'catalatically', that is, of decomposing hydrogen peroxide directly into water and oxygen. These studies have shed some light on the mechanisms of these reactions.

## Spectra of the Primary and Secondary Compounds

The colour of the three active or primary (I) enzyme-substrate compounds of catalase is pale green, and they have an absorption band at about 670 mμ. The Soret band is decreased in intensity and is slightly shifted towards the visible region of the spectrum, giving an isobestic point at about 435 mμ[5]. At 405 mμ the decrease of extinction coefficient per mole of hæmatin iron bound by peroxide is the same for the three primary complexes. Under suitable conditions the green compounds change into inactive or secondary (II) red compounds having two bands, at 536 and 572 mμ, approximately in the positions found by Stern[4] for catalase–ethyl hydrogen peroxide. The green-red shift of the hydrogen peroxide compounds superficially resembles that found by Theorell[6] for horseradish peroxidase and hydrogen peroxide. By analogy with his views, the green primary compounds are ferric iron peroxide complexes with ionic bonds, and the red secondary compounds are ferric iron peroxides with covalent bonds.

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## Molecular Composition of Catalase – Hydrogen Peroxide

In the primary compound of erythrocyte catalase and hydrogen peroxide three out of four hæmatin-iron groups of catalase can still react with cyanide ion or alkyl hydrogen peroxide. Thus the maximum concentration of catalase – hydrogen peroxide found under any condition corresponds to only one of the total number of hæmatins present in the catalase molecule. But the alkyl hydrogen peroxides combine with all four hæmatins, as is shown by the inability of their catalase compounds to combine with cyanide. This difference in composition of the primary hydrogen peroxide and alkyl hydrogen peroxide compounds causes a four-fold difference in the decrease of the intensity of the Soret band of catalase when these compounds are formed.

While a catalase in which all four hæmatin groups are combined with alkyl hydrogen peroxide is relatively stable except in the presence of alcohols, the attachment of more than one molecule of hydrogen peroxide to catalase results in an as yet undetected complex which rapidly decomposes. It is this 'catalatic' activity which limits the saturation value of the primary catalase – hydrogen peroxide complex to one hydrogen peroxide molecule per catalase molecule. This complex is the enzyme-substrate compound concerned in the decomposition of hydrogen peroxide, but it does not exhibit the properties of a Michaelis intermediate compound because the hydrogen peroxide not only unites with catalase to form the enzyme-substrate complex but also reacts again with this complex to cause the decomposition of hydrogen peroxide. Therefore, no Michaelis saturation effect is to be expected, and the velocity of destruction of hydrogen peroxide by strong catalase solutions is directly proportional to the hydrogen peroxide concentration, at least up to 0·3 $M$[7]. The previous evidence for a Michaelis constant of 0·025 $M$ appears to be an artefact due to the inactivation of catalase.

## Kinetics of Formation and Decomposition and the Enzyme-substrate Affinity

Of the three primary complexes, that with hydrogen peroxide forms the most rapidly ($k_1 \sim 3 \times 10^7$ $M^{-1}$ sec.$^{-1}$ (see ref. 5), that is, in a 1 $M$ hydrogen peroxide solution the complex would be half-formed in about $\frac{2 \cdot 3 \log 2}{3 \times 10^7}$ sec.) and the larger the peroxide molecule, the slower the reaction ($\sim 1 \times 10^6$ and $\sim 1 \times 10^4$ $M.^{-1}$ sec.$^{-1}$ for methyl and ethyl hydrogen peroxide respectively). This decrease is much greater than could be expected solely upon the basis of diffusion velocities and must be attributed mainly to a steric effect, due possibly to restricted accessibility of catalase hæmatin to these substances. In these three reactions, the kinetics of the formation of the primary complexes clearly represent the direct combination of catalase with peroxide without the formation of any detectable intermediates. The primary complexes are not stable; they decompose spontaneously into free catalase with a velocity constant of about 0·02 sec.$^{-1}$ (the half-time for the reaction is $\frac{2 \cdot 3 \log 2}{0 \cdot 02}$ sec.) even in the complete absence of alcohols.

Catalase shows the highest affinity for hydrogen peroxide $10^{-9}$ $M$ (that is, a steady-state concentration of about $10^{-9}$ $M$ hydrogen peroxide is required for half-saturation of the primary catalase – hydrogen peroxide complex). This high affinity is not obtained when hydrogen peroxide 'from the bottle' is added to catalase, for then the value is $1 \cdot 6 \times 10^{-6}$ $M$ (at a $3 \cdot 4 \times 10^{-6}$ $M$ catalase concentration[5]) due to the partial destruction of hydrogen peroxide during formation of the primary catalase – hydrogen peroxide complex. Catalase has an affinity equivalent to about $10^{-8}$ $M$ for methyl hydrogen peroxide and about $10^{-6}$ $M$ for ethyl hydrogen peroxide. These values are all increased in the presence of substances such as alcohols which cause the decomposition of these complexes.

The secondary compounds of catalase and these peroxides are formed from the primary compounds comparatively slowly, are much more stable than the primary compounds, and do not appear to react directly with alcohols. They are thus inhibitors of the catalytic activity of the primary complexes.

## Reactions of the Primary Complexes with Alcohols, etc.

Keilin and Hartree[9,10] have demonstrated that catalase and hydrogen peroxide or ethyl hydrogen peroxide can oxidize alcohols. These studies show that the primary catalase peroxide complexes are the active agent in these oxidations and are true Michaelis compounds since they fulfil the tests applied to the peroxidase – hydrogen peroxide complex[8]. The reaction-velocity constants for the reaction of the primary compounds with alcohols, etc. (calculated from the formulæ developed for peroxidase[8]), are very nearly independent of whether hydrogen peroxide or methyl or ethyl hydrogen peroxide is bound to catalase, and have values which decrease with the increasing size of the alcohol molecule, as is the case with the increasing size of the peroxide molecule. The velocity constant with ethanol or methanol is $k_4 \sim 1000$ $M^{-1}$ sec.$^{-1}$ (the turnover of the primary compounds in the presence of 0·001 $M$ ethanol is $1000 \times 0 \cdot 001$ times per sec.). The reactions of these three catalase peroxides with alcohols are not inhibited by carbon monoxide.

Direct spectroscopic measurements of the primary compound of catalase and hydrogen peroxide have been obtained in the presence of notatin, glucose, and oxygen. The absolute spectrum of the compound has been obtained with the Beckman spectrophotometer in the region 370–460 mμ and completely confirms the spectrum obtained using 'bottle' hydrogen peroxide and the rapid flow technique[5].

The direct spectroscopic studies of the turnover number of the catalase peroxides in presence of different alcohols agree with Keilin and Hartree's data obtained in coupled oxidations[10]. In such coupled oxidations the saturation of the catalase – hydrogen peroxide complex is found to be small in spite of the extremely high affinity of catalase for hydrogen peroxide. Therefore, the steady state hydrogen peroxide concentration during efficient coupled oxidations is only about $10^{-9}$ $M$. Only under these conditions is all the hydrogen peroxide utilized for alcohol oxidation and no molecular oxygen is evolved.

Two other biologically important substances have been found to react with catalase peroxides just as rapidly as do ethanol or methanol. These are formaldehyde (probably as methylene glycol) and formate. By a combination of the chromotrophic acid method[11] for formaldehyde production or disappearance and

an acidimetric method using brom-cresol purple for formic acid production and disappearance, the conversion of methanol to formaldehyde and formic acid, and the destruction of the latter, have been determined using ethyl hydrogen peroxide as the substrate. Similarly, carbon dioxide production from formate can be demonstrated in 'coupled oxidations' in which hydrogen peroxide is the substrate (Keilin and Hartree, personal communication).

The reaction of catalase – ethyl hydrogen peroxide with ethanol apparently gives acetaldehyde from ethanol and ethanol from ethyl hydrogen peroxide; the supply of ethanol is not at all depleted during the oxidation, and a small initial ethanol concentration may cause the reduction of a large amount of ethyl hydrogen peroxide. The destruction of strong ethyl hydrogen peroxide solutions by catalase studied by Stern[4], in which catalase is almost completely converted into the secondary compound, can be explained on the basis of a small percentage of the primary compound in equilibrium with the secondary compound. This primary compound reacts rapidly with ethanol present in ethyl hydrogen peroxide, and a small initial ethanol concentration will therefore cause the reduction of all the ethyl hydrogen peroxide. Thus the normal reactions of the primary compound were masked by the presence of Stern's inactive red complex.

The fact that catalase has a high affinity for ethyl hydrogen peroxide appears to contradict the results obtained by Stern[13], who failed to find appreciable inhibition of the destruction of hydrogen peroxide by catalase until about 0·1 *M* ethyl hydrogen peroxide solutions were used. However, these spectroscopic measurements show the 'stripping off' of alkyl hydrogen peroxide groups bound to catalase hæmatin upon the addition of hydrogen peroxide, with the result that only the catalase – hydrogen peroxide complex remains. In experiments in which hydrogen peroxide is continuously generated by notatin, glucose and oxygen, the rapid turnover of a large amount of catalase-bound alkyl hydrogen peroxide is observed spectroscopically. Thus hydrogen peroxide can take the place of an alcohol and can react with catalase alkyl peroxides. It does so with about the same velocity as it reacts directly with catalase. Therefore hydrogen peroxide has a reactivity of about 30,000 times $\left(\frac{3 \times 10^7}{10^3}\right)$ that of ethanol.

By a comparison of the activity of catalase-peroxides towards methanol, methylene glycol, and formic acid (all three are equally active) and ethanol, acetaldehyde, and acetic acid (the latter two are nearly inactive), it is concluded that both a hydroxyl group and a hydrogen atom attached to the one carbon atom are required of a suitable oxygen acceptor. The acceptor action of hydrogen peroxide constitutes an exception to this rule. Since the reaction velocity of both substrate and acceptor decreases with increasing size of the acceptor or substrate molecule, the acceptor molecule may have to pass through the same 'hole' as the substrate and may momentarily become attached to or near the catalase iron peroxide complex.

## Secondary Compound of Catalase and Hydrogen Peroxide

When catalase is continuously supplied with hydrogen peroxide by means of notatin, glucose, and oxygen, the slow conversion of the primary to the secondary complex ensues, and the two-banded spectrum (536 and 572 μ) may be seen in the hand spectroscope. Since these bands are close to those reported by Lemberg and Foulkes[14] for a compound of catalase and ascorbic acid, and since hydrogen peroxide formed by autoxidation of ascorbic acid has already been shown to give rise to the primary complex[5], there appears to be little evidence for the existence of this catalase – ascorbic acid complex.

The secondary complex has not yet been observed spectroscopically by adding 'bottle' hydrogen peroxide to catalase owing to the decomposition of the hydrogen peroxide in a fraction of a second. Under the conditions used in the determination of Kat *F*, catalase is in contact with 'bottle' hydrogen peroxide for ten minutes or more. The observed slow decrease of activity during this time has been proved to be due mainly to the formation of the secondary complex, since after the secondary complex has been formed by notatin, glucose, and oxygen, the diluted solution has only half the Kat *F* of the untreated solution. Furthermore, at *p*H 3·5 the rate of decrease of activity[7] and the rate of formation of the secondary complex in the presence of notatin, glucose, and oxygen are both accelerated. The extent of inactivation of catalase depends upon the number of catalase hæmatins converted to the secondary complex, and this varies with the experimental conditions.

This explanation of the inactivation of catalase by hydrogen peroxide justifies the new method for the determination of catalase activity, in which strong enzyme solutions are used so that the reaction is complete in a few minutes[7]. In this procedure there is insufficient time for the formation of the secondary complex and a nearly constant activity is therefore observed.

## Reaction Mechanisms

The nature of the reaction velocity constants obtained in these experiments suggests the following overall equations. The symbol "Cat" represents the four ferric-iron atoms of catalase hæmatin to which the peroxides become attached. The hydroxyl group found by Agner and Theorell[15] is considered to be replaced by the peroxide group. The equations are written in terms of methyl hydrogen peroxide and ethanol for simplicity, but similar reactions have been measured with ethyl hydrogen peroxide as substrate, and with several lower alcohols, formaldehyde, and formate in place of ethanol.

$$\mathrm{Cat(OH)_4 + 4CH_3OOH} \xrightarrow[<0{\cdot}01\ \mathrm{sec.^{-1}}]{1 \times 10^6\ M^{-1}\ \mathrm{sec.^{-1}}} \mathrm{4H_2O +\ Cat(-OOCH_3)_4 - I^* \longrightarrow Cat(-OOCH_3)_4 - II.} \quad (1)$$

$$\mathrm{Cat(-OOCH_3)_4 - I + 4C_2H_5OH} \xrightarrow{1100\ M^{-1}\ \mathrm{sec.^{-1}}} \mathrm{Cat(OH)_4 + 4CH_3OH + 4CH_3CHO.} \quad (2)$$

Thus the oxidation of alcohols by the primary catalase alkyl hydrogen peroxide complexes follows the simple theory of Michaelis and Menten, where $\mathrm{Cat(-OOCH_3)_4-I}$ is the Michaelis compound. The conversion of this primary compound into the secondary compound is negligible when the alcohol concentration is large and the peroxide concentration is small.

In Stern's experiments[4] the ethyl hydrogen peroxide concentration exceeded the ethanol concentration, and catalase was mainly in the form of the

secondary complex which he mistook for the active enzyme-substrate complex.

The oxidation of alcohols by the primary catalase hydrogen peroxide complex may also be regarded as following the Michaelis theory if proper allowance is made for the reactions of this complex with hydrogen peroxide (see equation 5) and for the fact that only one of the four catalase hæmatins is bound to hydrogen peroxide in this complex.

$$\mathrm{Cat(OH)_4 + H_2O_2} \underset{<0{\cdot}02\ \mathrm{sec.^{-1}}}{\overset{\sim 3\times10^7\ M^{-1}\ \mathrm{sec.^{-1}}}{\rightleftharpoons}} \mathrm{H_2O +}$$
$$\mathrm{Cat(OH)_3(-OOH) - I^* \rightleftharpoons Cat(OH)_3(OOH) - II.} \quad (3)$$

$$\mathrm{Cat(OH)_3(OOH) - I + C_2H_5OH} \xrightarrow{1000\ M^{-1}\ \mathrm{sec.^{-1}}} \mathrm{Cat(OH)_4 + CH_3CHO + H_2O.} \quad (4)$$

The overall mechanism for the destruction of hydrogen peroxide by catalase is represented :

$$\mathrm{Cat(OH)_4 + H_2O_2} \underset{<0{\cdot}02\ \mathrm{sec.^{-1}}}{\overset{\sim 3\times10^7\ M^{-1}\ \mathrm{sec.^{-1}}}{\rightleftharpoons}}$$
$$\mathrm{Cat(OH)_3(-OOH) - I^* \rightleftharpoons Cat(OH)_3(OOH) - II.} \quad (3)$$

$$\mathrm{Cat(OH)_3(OOH) - I + H_2O_2} \xrightarrow{\sim 3{\cdot}5\times10^7\ M^{-1}\ \mathrm{sec.^{-1}}} \mathrm{Cat(OH)_4 + H_2O + O_2.} \quad (5)$$

Although similar equations were previously proposed (for a summary see Sumner[16]), it is only now that they find experimental support. First, each step in equation 3 has been observed spectroscopically, and kinetic data have been obtained. Secondly, the reactions of $\mathrm{Cat(OH)_3(-OOH)-I}$ with alcohols according to equation 4 have been followed spectroscopically, as has the following reaction of catalase-bound alkyl hydrogen peroxide with hydrogen peroxide :

$$\mathrm{Cat(-OOCH_3)_4 + 4H_2O_2} \xrightarrow{3\times10^7\ M^{-1}\ \mathrm{sec.^{-1}}} \mathrm{Cat(OH)_4 + 4CH_3OH + 4O_2.} \quad (6)$$

It is therefore concluded that the second step of 'catalatic' activity is a second-order reaction of $\mathrm{Cat(OH)_3(-OOH)-I}$ with a second molecule of hydrogen peroxide according to equation 5. Thirdly, equations 3 and 5 indicate that, prior to the formation of appreciable amounts of the secondary complex, the overall velocity of decomposition of hydrogen peroxide will increase linearly with initial hydrogen peroxide concentration over a wide range. This is true at least up to 0·3 *M*, since the Michaelis constant of 0·025 *M* is an artefact[7]. In addition, the rapid and slow phases of catalase activity found by George[17] probably represent catalase activity in the absence and in the presence of the secondary complex.

In efficient coupled oxidations, the steady-state concentration of hydrogen peroxide is so low that the reaction velocity according to equation 5 is negligible compared with that according to equation 4. Therefore no molecular oxygen is evolved and hydrogen peroxide is utilized quantitatively in the oxidation of alcohol to aldehyde.

The reactions 1 and 2, 3 and 4, and 1 and 6, representing the 'peroxidatic' activities of catalase, can be analysed according to the Michaelis theory; but equations 3 and 5, representing the 'catalatic' destruction of hydrogen peroxide, cannot, since consecutive reactions of hydrogen peroxide with catalase and catalase–hydrogen peroxide are involved, and the saturation value of the catalase–hydrogen peroxide complex depends upon the relative velocities of these reactions. The formation of a catalase hydrogen peroxide complex containing one hydrogen peroxide molecule per four catalase hæmatins can be explained in two ways :

A purely 'kinetic' explanation is that the ratio of the velocity constant for the hydrogen peroxide reaction of equation 5 to that of equation 3 is 3 : 1 ; hence the maximum concentration of the catalase–hydrogen peroxide complex is a quarter the enzyme concentration. In this mechanism for equation 5, the hydrogen peroxide molecule may collide directly with the peroxide group attached to hæmatin or may collide with one of the three free catalase hæmatins to give, in either case, oxygen, water and free catalase. In the latter case, a catalase with only one hæmatin group would be 'catalatically' inactive (for a more detailed discussion see Theorell[18]). A second explanation assumes 'special properties' of the catalase–hydrogen peroxide complex. The existence of this complex is necessary for the decomposition of hydrogen peroxide into free oxygen and water or into radicals at the free catalase hæmatins, but the complex itself is not decomposed by these reactions. The complex may, however, decompose spontaneously or on reaction with alcohols, etc. In this case also a one-hæmatin catalase would be 'catalatically' inactive. These two mechanisms give very nearly identical mathematical solutions over the ranges of experimental conditions that can now be investigated with accuracy, and therefore a final conclusion on the exact mechanism for 'catalatic' activity is not yet possible.

A comparison of equations 2, 4 and 6 with equation 5 indicates that there is no fundamental difference between the 'peroxidatic' and 'catalatic' reactions ; 'catalatic' activity appears to be the special case of peroxidatic activity when the substrate and acceptor are identical.

Complete papers on these experiments will be published elsewhere.

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* The kinetics of this reaction do not follow first or second order kinetics with accuracy.

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