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On the Mechanism of the Decomposition of Hydrogen Peroxide by Catalase

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In a previous paper (Keilin and Hartree 1936) it was shown that catalase combines reversibly not only with KCN and H_2S as was shown by Zeile and Hellström (1930) or with C_2H_5OOH (Stern 1936) but also with NaN_3, NH_2OH, N_2H_4, NaF and NO, giving corresponding spectroscopically well-defined compounds.

In this respect and in the general pattern of the absorption spectrum, catalase shows a striking resemblance to acid methaemoglobin. It differs, however, from the latter in three important properties, namely:

1—Its catalytic activity is at least 1,000,000 times greater than that of methaemoglobin.

2—Its iron does not undergo reduction in presence of $Na_2S_2O_4$, which rapidly reduces methaemoglobin to haemoglobin.

3—Its absorption spectrum is not affected by the addition of H_2O_2 which undergoes an instantaneous, almost explosive decomposition, while with methaemoglobin it forms a well-defined compound (Keilin and Hartree 1935).

Of the compounds which react with catalase, sodium azide (NaN_3) and hydroxylamine are of special interest. They strongly inhibit the catalytic activity of the enzyme but do not appreciably change its colour or the general pattern of its absorption spectrum. The formation of these compounds is only recognized by changes in the position and intensity of some of the bands. However, on addition of traces of H_2O_2 to azide- or hydroxylamine-catalase, the colour of the solution turns from greenish-brown to red and the three-banded methaemoglobin-like absorption spectrum is replaced by two strong bands at 590 and 554 mμ. This compound combines reversibly with CO, giving a derivative with the bands at 580 and 545 mμ, and reverts to the original azide-catalase only in presence of molecular oxygen, which shows that its iron is in the divalent state.

It was shown previously (Keilin and Hartree 1936) that the reduced compounds, although autoxidizable, are not oxidized by $K_3Fe(CN)_6$ in complete absence of oxygen. This point seemed at first to contradict the evidence that the iron of azide- or hydroxylamine-catalase was actually reduced by peroxide. It was found, however, that these derivatives of catalase can be reoxidized to the initial ferric compounds by some other oxidizing agents such as $KMnO_4$, $CuCl_2$, somewhat more slowly by MnO_2 and very slowly by $HgCl_2$. It can be considered therefore as adequately demonstrated that azide- and hydroxylamine-catalase are reduced by H_2O_2 and reoxidized by molecular oxygen.

This property of azide-catalase makes it an excellent if not unique reagent for the detection of H_2O_2 where no other reagent can be used, for instance, during the aerobic oxidation of sodium hyposulphite ($Na_2S_2O_4$)* to sodium bisulphite. These experiments with azide- and hydroxylamine-catalase suggest that the normal catalytic activity of catalase is also accompanied by a similar change of valency of its iron, or in other words, the catalytic decomposition of H_2O_2 is brought about by the successive reduction of the catalase iron by H_2O_2 and its reoxidation by O_2 according to the equations (1) and (2), the sum of which (equation (3)) is the catalytic decomposition of H_2O_2 into molecular oxygen and water. This reversible

$$4Fe^{\cdot\cdot\cdot} + 2H_2O_2 = 4Fe^{\cdot\cdot} + 4H^{\cdot} + 2O_2 \quad (1)$$

$$4Fe^{\cdot\cdot} + 4H^{\cdot} + O_2 = 4Fe^{\cdot\cdot\cdot} + 2H_2O \quad (2)$$

$$2H_2O_2 = 2H_2O + O_2 \quad (3)$$

reduction is analogous to the reaction between ferric chloride and hydrogen peroxide, whereby the ferric ion is reduced and in the presence of $\alpha\alpha'$-dipyridyl gives rise to a pink compound. If the ferrous ion is not so fixed it becomes oxidized by molecular oxygen and all the peroxide is eventually decomposed.

The problem that arises now is whether the mechanism of the catalase reaction as shown in the above equations can be demonstrated experimentally.

As we have previously shown, the formation of an intermediate compound cannot be demonstrated spectroscopically, which indicates that the life of this compound is extremely short. Moreover, the main requirement for spectroscopic investigation is a very high concentration of enzyme, which

* In accordance with the recommendation of the International Union of Chemistry, we shall in future use the term "hyposulphite" in place of the original but misleading "hydrosulphite", when referring to $Na_2S_2O_4$ (Smith 1936).

in itself is particularly unfavourable for the detection of an intermediate compound.

On the other hand, if the catalytic decomposition of H_2O_2 requires the presence of molecular oxygen (as shown in equation (2)) for the reoxidation of the reduced compound, the reaction should be inhibited if the concentration of oxygen in the surrounding medium could be made sufficiently low. This can be achieved by having the reaction carried out with a very low concentration of catalase, which is possible owing to the very high activity of the enzyme. The volume of oxygen produced in this case by the direct reduction of catalase (equation (1)) would naturally be very small. If at the same time the reaction is carried out in an atmosphere of pure nitrogen and with vigorous shaking, in order to bring the greater part of this oxygen as rapidly as possible into the gas phase, the reaction (2) should be greatly slowed down and the catalase reaction thus inhibited. In fact, by carrying out experiments in pure N_2, H_2 or CO_2, and under conditions where there is no spontaneous decomposition of H_2O_2 due to shaking in an insufficiently cleaned glass vessel or to the presence of catalytic impurities, it has been found possible to inhibit the catalase reaction by as much as 99%.

Material and methods

Catalase preparation—Catalase used in this investigation was prepared from horse liver by the method previously described for the preparation A_3 (Keilin and Hartree 1936). This preparation contained varying amounts of a yellow-brown pigment with a general absorption towards the blue end of the spectrum. By a few slight modifications of the method, catalase solutions were obtained which were completely free from this pigment. These modifications can be summarized as follows:

1—The liver is preferably kept for 1 day or more at $-10°$ C. before mincing.

2—Minced liver is extracted for 24 hr. at room temperature, instead of 2 hr.

3—Instead of ultrafiltration, the elution from the calcium phosphate is treated with ammonium sulphate (450 g./l.), filtered, the precipitate suspended in water and electrodialysed until completely free from sulphate.

This method gives a distinctly greenish preparation (D) which differs from A_3 in being free from labile non-haematin iron. Unlike A_3 this preparation gives no change in colour on addition of $Na_2S_2O_4$ and $\alpha\alpha'$-dipyridyl or on treatment with H_2S. The ratio between total and haematin iron in this preparation is about 1·2, while that in fresh preparations of A_3 may be as high as 20. In other properties this preparation of catalase differs very little from A_3.

Calcium phosphate—The calcium phosphate used during the preparation of catalase was prepared as follows: 150 c.c. calcium chloride solution (132 g. $CaCl_2.6H_2O$ per litre) is diluted to about 1600 c.c. with tap water and shaken, with 150 c.c. trisodium phosphate solution (152 g. $Na_3PO_4.12H_2O$ per litre). The mixture is brought to *p*H 7·4 with dilute acetic acid and the precipitate washed three or four times by decantation with large volumes of water (15–20 l.). The precipitate is finally washed with distilled water in a centrifuge. 9·1 g. calcium phosphate is obtained.

Experimental conditions

The experiments are carried out at room temperature in Barcroft differential manometers. The left-hand flask receives 3·3 c.c. of phosphate solution, as under the conditions which will now be specified, the decomposition of H_2O_2 in absence of catalase is negligible. The right-hand flask receives about 1 c.c. of dilute catalase solution in glass distilled water, containing between 10^{-4} and $10^{-3}\gamma$ haematin and is made up to 3 c.c. with $M/30$ phosphate solution of pH 6·8 or simply $M/30$ acid phosphate. This flask contains also a dangling tube which receives 0·3 c.c. of 0·3 % H_2O_2. The flasks are evacuated and washed out several times with purified nitrogen and after equilibration of the temperature the taps of the manometer are closed, the manometer is read, the dangling tube is rapidly dislodged, mixing its content with that of the flask, and the manometers are rapidly shaken and read every minute.

In order to obtain clear and repeatable results the experiments must be carried out under well-defined conditions which can be summarized as follows:

1—The flasks, dangling tubes and all other apparatus must be carefully cleaned with chromic-sulphuric acid, thoroughly rinsed in tap water and in glass distilled water.

2—The dangling tubes, which receive H_2O_2, are 14 mm. long and 7 mm. in diameter, which latter dimension ensures rapid and complete mixing of its contents with that of the flask.

3—H_2O_2 used for the experiments is Merck perhydrol diluted 100 times with glass distilled water.

4—The amount of freshly diluted catalase used for each set of experiments must be finally fixed by preliminary experiments in order to give a rate of oxygen evolution not higher than 40 c.mm./min.

5—Phosphate buffer solutions are prepared from pure analytical reagents (KH_2PO_4 and Na_2HPO_4) dissolved in glass distilled water.

6—The manometers, after mixing enzyme and substrate, are shaken at a rate of about 200 times per minute.

7—The nitrogen from a cylinder must be purified over hot copper.

Results of the experiments

The results of these experiments which are summarized in fig. 1 and Table I clearly demonstrate that the removal of oxygen inhibits the catalytic

activity of the enzyme dissolved in acid phosphate solution by 88–99%. For all practical purposes, we can say that in complete absence of molecular oxygen the catalytic activity of the enzyme is completely abolished. In fact, a very slight activity shown in these experiments is due only to traces of oxygen which are still present in the surrounding medium. Moreover, the incomplete inhibition (40–70 %) of the same reaction at *p*H 6·8 is due

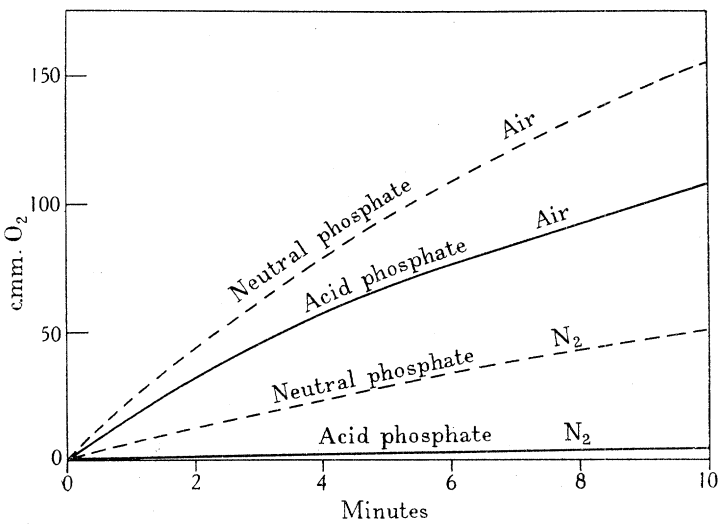

FIG. 1—Effect of pure N_2 on the decomposition of H_2O_2 by a purified catalase preparation: Catalase D (diluted to approximately 10^{-9} M with respect to haematin); 0·3 c.c. 0·3 % H_2O_2; 0·03 M phosphate buffer; $T = 19°$ C.

TABLE I

Exp. no.	Catalase	Phosphate buffer	O_2 evolved c.mm./min. In N_2	O_2 evolved c.mm./min. In air	% inhibition due to absence of O_2
1	A_3	*p*H 6·8	17	29	41
2	A_3	*p*H 6·8	6·5	16·5	61
3	A_3	*p*H 6·8	18	42	57
4	A_3	*p*H 6·8	3·5	11	68
5	A_3	KH_2PO_4	0·3	13	98
6	A_3	KH_2PO_4	0·5	14·5	97
7	D	KH_2PO_4	0·5	8	94
8	D	KH_2PO_4	0·3	16·5	99
9	D	KH_2PO_4	0·7	6	88
10	E	KH_2PO_4	5·0	17	71
11	F	KH_2PO_4	3·0	32	91

A_3 and D, catalase preparations previously described; E, crude extract of germinating cucumber seeds; F, crude liver extract.

to a slight non-enzymic decomposition of H_2O_2 (15 c.mm. O_2/hr.) sufficient for a partial activity of the enzyme. A similar effect is noticeable in the case of preparation E, where catalytic impurities are present.

Effect of carbon monoxide

It was demonstrated spectroscopically that azide-catalase reduced by H_2O_2 combines with CO and it was shown manometrically that the decomposition of H_2O_2 by catalase is accompanied by the reduction of its iron. These results suggest the possibility that CO may act as inhibitor of the normal catalytic reaction.

Califano (1934), using a manometric method, claimed that the decomposition of H_2O_2 by catalase in CO was strongly inhibited when compared with the reaction taking place in N_2 and that this inhibition was much reduced or completely abolished by light. These results, however, were not confirmed by other authors.

Our results with the reaction carried out in purified N_2 indicate the possibility of some confusion in the work of previous authors. In fact, inhibition by CO may be due either to the absence of O_2, CO acting only as an inert gas, or to its combination with the divalent iron of reduced catalase. Thus, the former inhibition would occur only in purified CO and would not be reversed by light, whereas the latter would occur in a CO : O_2 mixture and would be affected by light. We have now shown that under conditions where purified N_2 strongly, if not completely, inhibits the catalase reaction, purified CO exerts a similar non light-sensitive effect (Table II).

Table II

Exp. no.	Catalase	Buffer	O_2 evolved c.mm./min. CO (light)	CO (dark)	Air
1	A_3	KH_2PO_4	0	0	22
2	D	KH_2PO_4	0	1	14
3	From blood	KH_2PO_4	0	0	13
4	From blood	KH_2PO_4	—	0	14

In these experiments the inhibition due to the absence of oxygen is even greater than in the previous experiments using pure nitrogen. It can be supposed that, in absence of oxygen, there is no competition for the reduced catalase and consequently the complete inhibition observed is the sum of the "nitrogen effect" and a "CO effect". On the other hand, the experiments carried out in non-purified CO containing about 0·3% O_2, or in a gas

mixture CO : O_2 = 4 : 1, using the reaction in air as a standard, did not give consistent results. It can be stated however that the purest samples of catalase are not inhibited by CO in the dark in presence of a small concentration of O_2. This shows that the reduced catalase has a much greater affinity for oxygen than for CO.

However, during the preparation of catalase A_3, fractions have frequently been obtained showing a variable but very distinct light sensitive inhibition by CO (Table III). Unfortunately, such preparations could not be obtained at will and the sensitivity of the catalase to CO in such preparations was probably due to some impurities affecting the relative affinities of the reduced catalase for CO and O_2. This supposition is supported by the fact that in some crude liver extracts CO inhibits the catalytic reaction by 40–50% (F, Table III).

TABLE III

Exp. no.	Catalase	Buffer	c.mm. O_2 evolved per min. CO.L.	CO.D.	$CO/O_2 = 4$	Air
1	A_3	KH_2PO_4	—	—	8	16
2	A_3	KH_2PO_4	—	—	12	13
3	A_3	KH_2PO_4	—	—	5·5	9
4	A_3	KH_2PO_4	14	6	—	11
5	A_3	KH_2PO_4	10	6	—	11
6	A_3	KH_2PO_4	25	16	—	26
7	E	KH_2PO_4	—	—	12·5	17
8	F	KH_2PO_4	—	—	14	26

CO.L. and CO.D. non-purified CO containing about 0·3% O_2 in light and dark; E, crude extract of germinating cucumber seeds; F, crude extract of horse liver.

Moreover, preparations of catalase D, which are never affected by CO, can be made sensitive to this gas in the dark by adding traces of various substances such as sodium azide, cysteine and glutathione. The inhibition of the catalase reaction produced by CO under these conditions is completely reversible in light (figs. 2, 3).

The radiation used in these experiments was the visible region of a mercury-vapour lamp, which was shown to be without effect on the catalase reaction in gases other than CO.

SUMMARY AND CONCLUSIONS

1—A new explanation of the mechanism of the decomposition of H_2O_2 by catalase is proposed. According to this explanation the reaction is brought about by the successive reduction of the catalase iron by H_2O_2 and its

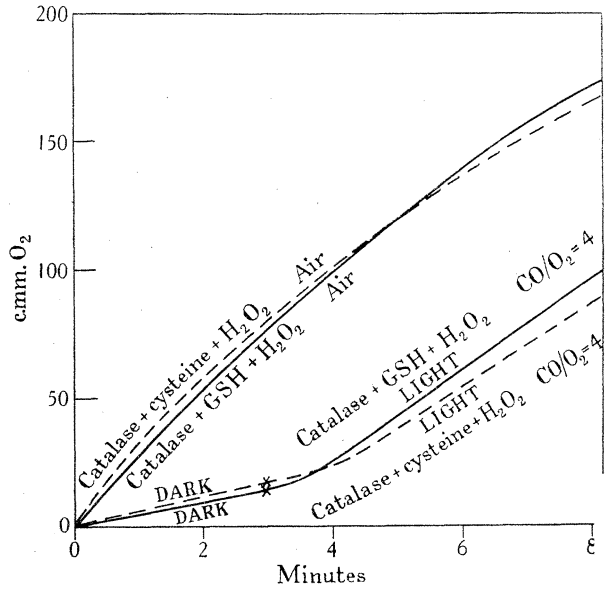

FIG. 2—Effect of CO in dark and light on catalase D (approx. 10^{-9}M haematin) in presence of 10^{-4}M glutathione and 10^{-4}M cysteine; 0·03M KH_2PO_4; $T = 19°$ C.

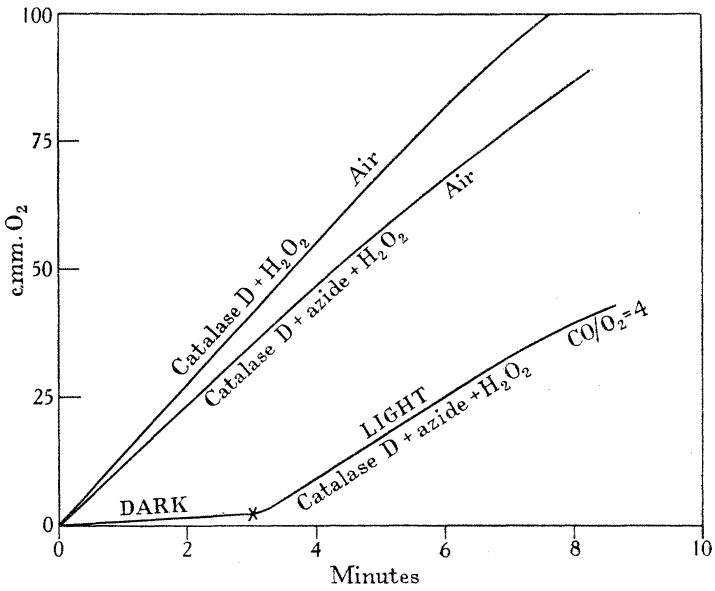

FIG. 3—Effect of CO in dark and light on catalase D (10^{-9}M haematin) in presence of sodium azide (10^{-7}M); 0·03M KH_2PO_4; $T = 19°$ C.

reoxidation by molecular oxygen; the reaction proceeding according to the following equations:

$$4Fe^{\cdot\cdot\cdot} + 2H_2O_2 = 4Fe^{\cdot\cdot} + 4H^{\cdot} + 2O_2$$
$$4Fe^{\cdot\cdot} + 4H^{\cdot} + O_2 = 4Fe^{\cdot\cdot\cdot} + 2H_2O$$
$$2H_2O_2 = 2H_2O + O_2$$

2—This explanation is supported by the following experiments:

a—It has been shown manometrically that the decomposition of H_2O_2 by catalase does not proceed in absence of molecular oxygen.

b—It has been shown spectroscopically with azide- and hydroxylamine-catalase that H_2O_2 is the only known substance capable of reducing catalase iron, which can then be readily reoxidized by molecular oxygen.

3—The inhibitors of catalase can be separated into two groups, those like KCN, H_2S, C_2H_5OOH which inhibit the reduction of catalase iron by H_2O_2 and those like azide, hydroxylamine and hydrazine which inhibit the reoxidation of the reduced catalase by molecular oxygen (Keilin and Hartree, 1936).

4—The decomposition of H_2O_2 by pure catalase preparations is not inhibited by CO in presence of even a very small concentration of oxygen, which shows that the reduced catalase has a much greater affinity for oxygen than for CO.

5—Some catalase preparations have been obtained which show a more or less marked light sensitive inhibition by CO. Pure catalase preparations become sensitive to CO in presence of a very small concentration of azide, cysteine or glutathione, substances which apparently inhibit the reoxidation of reduced catalase.

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