SUMMARY

1. A direct-reading flame photometer for the estimation of sodium and potassium in urine and serum is described, which has been in continuous use for six years.

2. The flame spectrum has been analysed and the efficiency of the filter combinations assessed.

3. The operation of diluting and reading of samples containing 2–5 $\mu\text{g.}$ sodium or potassium/ml. has a mean standard deviation of 0.03 $\mu\text{g./ml.}$

4. The validity of sodium and potassium estimations in urine and serum, by direct comparisons with standard solutions of sodium or potassium chloride, has been assessed by addition experiments, comparison with chemical methods and serial dilutions.

5. Urinary sodium and potassium estimations are free from interference at sample dilutions between 1:300 and 1:1000. At lower dilutions readings of both sodium and potassium are depressed.

6. Serum sodium estimations are free from interference, but serum potassium estimations (dilution 1:100) are subject to a systematic error of +13 % of the chemical estimation. Estimations accurate to $\pm 1.5\%$ can, however, be made by applying an arithmetical correction. At lower dilutions, progressive depression of the potassium reading occurs.

We would like to record our indebtedness to Dr G. H. Beaven for much valuable assistance, to Miss E. H. Aitken for technical help, and to Prof. C. Wilson for laboratory facilities.

REFERENCES

- Aitken, E. H. & Preedy, J. R. K. (1953). *Biochem. J.* **55**, 211.
 Barnes, R. B., Richardson, D., Berry, J. W. & Hood, R. L. (1945). *Industr. Engng Chem. (Anal.)*, **17**, 605.
 Crismon, J. M. (1948). *Fed. Proc.* **7**, 24.
 Domingo, W. R. & Klyne, W. (1949). *Biochem. J.* **45**, 400.
 Dreguss, M. (1939). *Biochem. Z.* **303**, 69.
 Gilbert, P. T., Hawes, R. C. & Beckman, A. O. (1950). *Analyt. Chem.* **22**, 772.
 Hald, P. M. (1947). *J. biol. Chem.* **167**, 499.
 Kapuscinski, V., Moss, N., Zak, B. & Boyle, A. J. (1952). *Amer. J. clin. Path.* **22**, 687.
 Kiers, R. J. & Speck, S. J. (1950). *J. Dairy Sci.* **33**, 413.
 Mosher, R. E., Boyle, A. J., Bird, E. J., Jacobson, S. D., Batchelor, T. M., Iseri, L. T. & Myers, G. B. (1949). *Amer. J. clin. Path.* **19**, 461.
 Overman, R. R. & Davis, A. K. (1947). *J. biol. Chem.* **168**, 641.
 Rieben, W. K. & Van Slyke, D. D. (1944). *J. biol. Chem.* **156**, 743.
 Ryssing, E. (1951). *Scand. J. clin. Lab. Invest.* **3**, 17.
 Spencer, A. G. (1950). *Lancet*, **259**, 623.
 Tenery, R. M. & Anderson, C. E. (1940). *J. biol. Chem.* **135**, 659.
 Wallace, W. M., Holliday, M., Cushman, M. & Elkington, J. R. (1951). *J. Lab. clin. Med.* **37**, 621.
 Wynn, V., Simon, S., Morris, R. J. H., McDonald, I. R. & Denton, D. A. (1950). *Med. J. Aust.* **1**, 821.

The Chemical Nature of the Second Hydrogen Peroxide Compound formed by Cytochrome c Peroxidase and Horseradish Peroxidase

2. FORMATION AND DECOMPOSITION

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Spectrophotometric titration of the second intermediate compound formed by horseradish peroxidase or cytochrome c peroxidase and hydrogen peroxide, using the reducing agents ferrocyanide ion, ferrous ion and ferrocycytochrome c showed that this intermediate compound is one oxidation equivalent above the original ferric state of the enzyme (George, 1952*a*, *b*, 1953), e.g.

This compound can therefore be regarded as containing iron with an effective oxidation number of +4, and it differs in two important respects from the

classical type of 'enzyme-substrate complex' with which it has long been identified (Chance, 1943, 1949*a-c*, 1951*a, b*). First, the substrate, hydrogen peroxide, is not a component part of the structure, because the intermediate is only one oxidation equivalent, and not two, above the ferric state of the enzyme. Secondly, the intermediate cannot dissociate to give the free enzyme and a valency saturated product: it can only be reduced to the free enzyme.

In this paper experiments on the formation and decomposition of the second intermediate compound are described which reveal a further feature

in which it differs from an enzyme-substrate complex. It is not formed from peroxidase and peroxide in an equilibrium system but in a series of irreversible reactions competing for the oxidizing capacity of the peroxide.

METHODS

Symbols. Horseradish peroxidase and cytochrome *c* peroxidase are abbreviated to HRP and CcP.

E_{425} and ΔE_{425} represent the optical density of a solution at 425 m μ . and the optical density increment observed when a reaction takes place. Measurements at other wavelengths are indicated by the appropriate subscript.

Reactions. Reactions were carried out at room temperature, 28–32°, in 1 or 5 cm. cuvettes of a Beckman Model DU spectrophotometer and optical densities measured in the usual way. 1 cm. cuvettes were used unless otherwise stated.

Preparations. Methyl hydroperoxide was prepared according to Baeyer & Villiger (1901) and estimated by iodometric titration. Other experimental details including the preparative methods for the peroxidases, determination of their concentrations, etc., have been fully described in Part I (George, 1953). For the complete formation of compound II with HRP and CcP, $\Delta\mu\epsilon_{425}$ has been taken as before to be 0.044 and 0.0405 respectively. The value for CcP includes a correction for the fact that diluent protein in the enzyme preparation gave values for its concentration determined from E_{410} about 8% too high, this figure being estimated from equilibrium measurements on its cyanide complex. Four samples of CcP, referred to as sample 1, sample 2, etc., were used, and four of HRP. One of these had been prepared by Theorell's method (sample 1) and was that employed in part I: the other three, which were kindly supplied by Dr E. F. Hartree, had been prepared by Keilin & Hartree's more recent procedure (1951). Samples 2 and 3 had been prepared for a few months and sample 4 had been stored for 2 years.

RESULTS

Titration of horseradish peroxidase and cytochrome c peroxidase with hydrogen peroxide

The purpose of these experiments was to determine how the amount of compound II formed depends on the peroxide concentration and to see whether the data support a reaction mechanism in which compound II is in equilibrium with free enzyme and peroxide.

Varying amounts of a 27 μM - H_2O_2 solution were added to an approximately 1.0 μM -HRP solution, sample 1, in 0.01 M-phosphate buffer, pH 5.3, to give peroxide concentrations ranging from 0.2 to 2.0 μM . With the fresh enzyme sample the half-life of compound II was about 5 min., so, to get the amount of compound II formed initially, optical density changes at 390, 395, 400, 420, 425, and 430 m μ . were followed for 12 min. and the curves extrapolated to zero time. The density changes at the three shorter wavelengths gave values for the amount of compound II formed about 14% lower than those obtained at the longer wavelengths. The

discrepancy is probably due to small amounts of compound I being present which would have this effect: this is supported by the observation that even with a three- to ten-fold molar excess of peroxide present only 80% of compound II was formed in the absence of added reducing agents like *p*-cresol. For this reason the three higher wavelength values have been taken as the more reliable; the percentages of compound II formed were worked out on the basis that at 425 m μ . an optical density change of 0.044 accompanies the formation of a 1 μM solution. The results are shown in Fig. 1 where the percentage formation is plotted as a function of the H_2O_2 /HRP concentration ratio.

Fig. 1. Titration of HRP with H_2O_2 giving percentage compound II as a function of the H_2O_2 /HRP concentration ratio at pH 5.35. □, First titration, 1.0 μM -HRP; Δ, second titration, 0.20 μM -HRP in 5 cm. cuvette; ○, third titration, 1.0 μM -HRP.

The results of a similar titration carried out a few months later, when compound II was found to be much more stable in the system, is also shown together with results of a titration carried out in 5 cm. cuvettes using HRP and H_2O_2 solutions of one-fifth of the previous concentrations, i.e. 0.2 μM -HRP and 0.02–0.25 μM - H_2O_2 .

The samples of HRP prepared by Keilin & Hartree's method (1951) gave substantially the same results, except that even less compound II was formed with excess peroxide present. With the fresh samples, 2 and 3, 70 and 60% were formed, respectively. At low values of the H_2O_2 /HRP ratio, compound II began to decompose as soon as it was formed and optical densities were plotted against time so that the amount formed initially could be estimated. This was taken to correspond to the extrapolated value 2 sec. from the start of the reaction to make adequate allowance for Chance's (1949b) finding that compound II formation can take 0.5 sec. for completion under similar condi-

tions. Similar titration curves to those in Fig. 1 were obtained with both samples, although the extent to which the curve lay above the line corresponding to the formation of 1 mole of compound II from 1 mole of H_2O_2 was less marked. With the old sample, 4, only 50% compound II was formed with excess H_2O_2 present, and the titration curve lay well below the line. This appeared to be due to less reducing matter being present to bring about the reduction of compound I to II, because the addition of a further amount of peroxide to an enzyme solution in which compound II had been formed and allowed to decompose gave even less compound II than before.

Fig. 2. Titration of CcP sample 2 with H_2O_2 giving percentage compound II as a function of the $\text{H}_2\text{O}_2/\text{CcP}$ concentration ratio at pH 5.35. Δ , $0.24 \mu\text{M}$ -CcP; $\odot$, $1.20 \mu\text{M}$ -CcP in 5 cm. cuvette; $\square$, $4.85 \mu\text{M}$ -CcP.

Using CcP sample 2 a titration carried out in the same manner at three different concentrations of CcP, 4.85, 1.20 and $0.24 \mu\text{M}$, gave the results shown in Fig. 2. The percentage compound II formation was obtained from optical density increments measured at 420, 425, 430 and 435 $\text{m}\mu$. on the basis that $\Delta\epsilon_{425}^{425}$ is 0.0405 for the formation of compound II. The points obtained using $1.2 \mu\text{M}$ -CcP solution show greater scatter than the others; the reason for this is not known but it was accompanied by similar variations in the maximum optical density changes obtained when excess peroxide was added, such that if the observed change divided by the maximum change is used to calculate the percentage compound II formed the points lie on a much smoother curve.

An attempt was also made to obtain a titration curve at a much higher CcP concentration of $27.5 \mu\text{M}$ by following the formation of compound II by measuring optical densities at 560 $\text{m}\mu$. where compound II has a strong absorption band. But compound II was found to be too unstable under these conditions, with a half-life of about 1 min.

Similar titration results to those given in Fig. 2 were obtained with the three other CcP preparations and, with sample 1, titrations carried out at pH 5.3 and 7.0 were found to give identical results as shown in Fig. 3. With this sample the greatest difference in behaviour was found, a ratio of $\text{H}_2\text{O}_2/\text{CcP}$ of the order of 1.2 to 1.3 being required for the full formation of compound II, as compared to a ratio of 0.7 to 0.95 for the other three samples.

There is clear evidence that under these experimental conditions more than one mole of compound II can be formed for 1 mole H_2O_2 , especially at low $\text{H}_2\text{O}_2/\text{peroxidase}$ concentration ratios.

These titration results do not agree with those of previous workers (e.g. Altschul, Abrams & Hogness, 1942) who found the titration curve to lie along and below the line corresponding to the formation of 1 mole compound II from 1 mole H_2O_2 . The difference

Fig. 3. Titration of CcP sample 1 with H_2O_2 and CH_3OOH giving percentage compound II as a function of the $\text{H}_2\text{O}_2/\text{CcP}$ or $\text{CH}_3\text{OOH}/\text{CcP}$ concentration ratio. $1.0 \mu\text{M}$ -CcP used. $\square$ and $\odot$, H_2O_2 titrations at pH 5.3 and 7.0 respectively; $\bullet$, CH_3OOH titration at pH 5.3.

appears to be outside any experimental uncertainties, for instance the precise value for $\Delta\epsilon_{425}^{425}$. A possible explanation is that they used a much higher concentration of enzyme that may have contained more reducing matter, thus affecting the formation of compound II, and that slight decomposition of compound II also occurred.

Titration of CcP with CH_3OOH

Within experimental error the spectrum of compound II obtained with CH_3OOH was identical with that for H_2O_2 and the titration curve, shown in Fig. 3, had the same general characteristics indicating that more than 1 mole of compound II can be formed from 1 mole of CH_3OOH .

A test whether compound II is formed in an equilibrium system

In general, a titration curve of the type shown in Figs. 1-3 could arise from one of two mechanisms, either a rapidly established equilibrium where the

Table 1. *The addition of more CcP to a solution of CcP with just enough H₂O₂ present to give 95–100 % compound II*

(7.6 μ M-CcP stock solution used in 0.01M-phosphate buffer, pH 5.35 at 30°. *E* has been corrected for dilution where necessary to bring all volumes to correspond to a total volume of 2.5 ml. *A*5 is calculated from *A*1. *B*4 is obtained by subtracting *A*2 from *B*2.)

Reference	Particulars of solution, or ΔE	Optical density measurements ($\times 10^3$) at			
		420 m μ .	425 m μ .	430 m μ .	435 m μ .
<i>A</i> 1	CcP solution alone, 0.25 ml. in 2.5 ml.	56	43	32	24
2	After addition of 0.05 ml. 50 μ M-H ₂ O ₂	79.5	74.5	60	43
3	After addition of 0.10 ml. 7.6 μ M-CcP	102	92.5	72	53
4	ΔE resulting from this CcP addition	22.5	18	12	10
5	Calculated ΔE on the basis that the CcP does not react	22.5	17	13	9.5
<i>B</i> 1	CcP solution alone, 0.35 ml. in 2.5 ml.	78	59.5	43.5	35.5
2	After addition of 0.05 ml. 50 μ M-H ₂ O ₂	103.5	96	75.5	55
3	Density resulting from same H ₂ O ₂ addition to previous CcP solution, i.e. <i>A</i> 2	79.5	74.5	60	43
4	ΔE if H ₂ O ₂ is present initially with CcP	24	21.5	15.5	12
5	Observed ΔE if CcP is added in two portions, i.e. <i>A</i> 4	22.5	18	12	10
<i>C</i> 1	Calculated <i>E</i> for 85 % compound II in CcP solution, 0.35 ml. in 2.5 ml.	103.5	95.5	76.5	55.5
2	Observed <i>E</i> in <i>B</i> 2 above	103.5	96	75.5	55

affinity of the enzyme for the peroxide is not high enough to give 100 % formation at a H₂O₂/peroxidase concentration ratio of 1, or a set of competing irreversible reactions involving at some stage competition for the peroxide or an oxidizing intermediate between the ferric iron atom and other groups such that the full oxidizing capacity of the peroxide does not appear finally in the amount of compound II formed. An experiment to decide between these two possibilities was carried out by adding a further small quantity of concentrated CcP to a solution made from CcP and H₂O₂ where the concentration ratio was chosen to give just 100 % compound II formation. If equilibrium conditions prevail then there should have been either 26 or 63 % free H₂O₂ present according to the titration curve, depending on whether 1 mole of H₂O₂ forms 1 or 2 moles of compound II, and so further addition of CcP should have resulted in the formation of more compound II. If the system is not in equilibrium then there should have been no peroxide left and addition of CcP should have produced no more compound II. Table 1 gives the results of the experiment in which optical density measurements were made at 420, 425, 430 and 435 m μ . A comparison of the optical densities in lines *A*4 and *A*5 show that within experimental error the added CcP did not react to form any additional compound II. A check on the validity of this result is given by a second experiment in which peroxide was added to the combined amounts of CcP: a comparison of lines *B*4 and *B*5 show that more compound II was formed under these conditions.

Finally in lines *C*1 and *C*2 the results of a calculation are given showing that this last result is in

accord with the appropriate titration curve, where, with the operative concentration ratio of H₂O₂/CcP 85 % compound II should have been formed. The curve referred to here is one based on the optical density increment for 100 % compound II formation determined for this particular enzyme sample and not on the mean value used elsewhere. This procedure is clearly essential in a calculation of this kind.

The effect of ferrocyanide ion, present initially, on the formation of compound II

It can now be appreciated that compound II resembles the metmyoglobin-peroxide compound in two important chemical aspects. Both compounds are reducible to the ferric haemoprotein in a one-equivalent step, and both are formed from peroxide in non-equilibrium systems resulting in the loss of some of the oxidizing capacity originally present in the peroxide if only the actual amount of intermediate compound formed is considered. With metmyoglobin, George & Irvine (1951, 1952) found that ferrocyanide, added initially, reacted with a transient oxidizing species possessing one oxidizing equivalent, which was produced along with the metmyoglobin intermediate compound, and that on this basis the full oxidizing capacity of the peroxide could be accounted for.

Results of similar experiments carried out with CcP are given in Fig. 4, where the optical density of the solution at 425 m μ . is plotted against time for three solutions in which peroxide was added to give 0.80 μ M with 0.79 μ M-CcP and 0.33, 0.65 and 1.08 μ M-ferrocyanide, respectively. The rapid fall in optical density observed shows clearly that only a little

ferrocyanide is used up during the formation of compound II and that the greater part remains to bring about its reduction. The initial concentration of compound II has been obtained by extrapolation as shown in Fig. 4; together with the final concentration this value has been used to calculate the ferrocyanide used in the subsequent reduction of compound II. Subtraction from the concentration

Fig. 4. Optical density changes at 425 $m\mu$. when H_2O_2 to give a $0.80 \mu M$ -solution is added to a mixture of $0.79 \mu M$ -CcP and (a) $0.33 \mu M$ -, (b) $0.65 \mu M$ - and (c) $1.08 \mu M$ -ferrocyanide, respectively, at pH 5.3.

of ferrocyanide initially present gives that used up during the formation of compound II, and thus an estimate of the oxidizing capacity of the peroxide which can be accounted for by these two processes. Table 2 shows that with the highest ferrocyanide concentration only 74% of the peroxide is accounted for. Higher concentrations could not be employed because they would have completely reduced all the compound II making the calculation impossible. There is thus a marked quantitative difference between the reactions of metmyoglobin and CcP with hydrogen peroxide which is probably due to the participation of compound I in the latter

reaction. This will be discussed later. There can be little doubt, however, that ferrocyanide does compete in the reactions leading to the formation of compound II, for the amount which reacts is approximately proportional to its initial concentration.

Fig. 5. Plots showing the first-order kinetics of the decomposition of CcP compound II at 30° obtained from optical density measurements at $560 m\mu$. after several successive amounts of H_2O_2 had been added and allowed to decompose in the solution. The lines a, b and c are for the first three experiments, respectively, listed in Table 3 where full details are given.

The spontaneous decomposition of CcP compound II

As mentioned earlier, the titration of $27.5 \mu M$ -CcP with H_2O_2 was found to be impracticable because compound II decomposed rapidly. However, it was possible under these conditions to determine whether this spontaneous decomposition was affected by several additions of peroxide as Chance (1949b) observed with HRP. The decomposition was found to be a first-order reaction as shown in Fig. 5, where $\log_{10} \Delta E_{560}$ plotted against time is

Table 2. The effect of adding H_2O_2 to give $0.80 \mu M$ -solution in a mixture of CcP and ferrocyanide, observed by measuring optical density changes at $425 m\mu$.

(All concentrations in μM . The data in columns 2 and 3 are obtained from the curves in Fig. 4.)

Total ferrocyanide concentration	Compound II formed initially	Compound II reacting subsequently with ferrocyanide	Difference between concentrations in columns 1 and 3	Oxidizing equivalents accounted for
0.33	0.67	0.21	0.12	0.79 (49%)
0.65	0.59	0.29	0.36	0.95 (59%)
1.08	0.50	0.39	0.69	1.19 (74%)

Table 3. *First-order decomposition velocity constants at 30° for CcP compound II, after several successive amounts of H₂O₂ had been added and compound II allowed to decompose, obtained from optical density measurements at 560 m μ .*

(Originally there were 0.0686 μ moles CcP present in 2.5 ml. 0.01 M-phosphate buffer, pH 7.0 (i.e. 27.5 μ M-CcP); dilution by the peroxide brought the final volume up to 3.02 ml. The kinetic plots for the first three velocity constants are given in Fig. 5 (a-c) respectively.)

No. of successive H ₂ O ₂ additions	μ moles H ₂ O ₂ previously added and decomposed	μ moles H ₂ O ₂ at start of kinetic experiment	μ moles H ₂ O ₂ decomposed at end of experiment = p	Ratio: p/μ moles CcP	$k \times 10^3 \text{ sec.}^{-1}$
6	0.156	0.12	0.276	4.0	1.15
8	0.396	0.12	0.516	7.5	1.05
11	0.756	0.12	0.876	12.7	0.96
12	0.876	0.60	1.48	21.5	1.06
14	2.08	0.60	2.68	39	0.95
16	3.68	1.00	4.68	68	0.82

linear, ΔE_{560} being a measure of the compound II remaining at any time. In all, sixteen additions of peroxide were made, and compound II was allowed to decompose almost completely (97 %) before the next addition. Finally, 4.68 μ moles H₂O₂ had been added and decomposed in the solution compared to the 0.0686 μ mole CcP present. Table 3 gives a selection of the first-order constants obtained throughout the series of additions showing that there is only a very slight indication that compound II became more stable.

The effect of fluoride and cyanide ions on the stability of compound II

Chance (1952a) has observed that when sodium fluoride is added to a solution of HRP and excess H₂O₂ which shows the spectrum of compound II, this spectrum is replaced by that of compound I. In general, two types of explanation could be advanced. First, that the fluoride alters rapidly reversible equilibrium conditions in the system such that a new state of equilibrium is reached favouring a high concentration of compound I rather than compound II. Secondly, that in a non-equilibrium system the transition from compound I into compound II becomes rate limiting in the presence of fluoride.

Inherent in the second explanation is a loss of oxidizing capacity in the system, that is destruction of peroxide accompanying the decomposition of compound II, and either an increase in the decomposition rate of compound II in the presence of fluoride or a decrease in the rate of formation of compound II from compound I. The following experiments carried out with CcP support the second type of explanation.

(1) Preliminary tests with CcP and fluoride at pH 5.3 showed that about 200 μ M-sodium fluoride was required to give full conversion of CcP into its fluoride complex, the spectrum of which was given previously (George, 1953). The addition of fluoride to a 0.82 μ M-CcP solution, containing 176 μ M-H₂O₂ at pH 5.3 to give 7.35 mM-sodium fluoride,

resulted in the absorption band at 420 m μ . decreasing in intensity and spreading to the shorter wavelength side characteristic of compound I. This confirms that CcP undergoes the same change that Chance observed with HRP: but reduction with ferrocyanide gave evidence that considerable enzyme destruction also occurs, for a very diffuse band of high intensity was obtained, unlike the sharp band characteristic of the CcP-fluoride complex at 407 m μ . This destruction indicates that the enzyme is going through an oxidation-reduction cycle and that no mere shift of an equilibrium has occurred.

(2) When a concentrated fluoride solution was added to CcP containing just sufficient H₂O₂ to convert all the CcP into compound II with a resulting fluoride concentration of 170 μ M, rapid decomposition of compound II occurred, and by 7 min. complete conversion into the fluoride complex had taken place. The addition of a further portion of peroxide equal to that used initially gave momentarily about 50 % of compound II which then decomposed rapidly as before.

(3) Using a lower fluoride concentration the full formation of compound II could be observed even in a solution where its subsequent decomposition was still rapid. This is shown in Fig. 6 where the optical density at 420 m μ , the isobestic point for CcP and its fluoride complex, is plotted against time. To a solution containing 0.76 μ M-CcP and 53 μ M-sodium fluoride, concentrated peroxide was added at intervals giving 1.2, 0.8 and 1.2 μ M-H₂O₂ in the cuvette. The curves in Fig. 6 show that the transitory formation of compound II was 100, 85 and 100 % respectively, in good agreement with the previous titration of the CcP sample which gave 100, 90 and 100 % for these concentrations. At pH 7.0 similar results were obtained except that slight destruction of the CcP was observable by a drop in the final optical density of the solution.

It is clear from these experiments that when the minimal amount of H₂O₂ has been added to convert the CcP into compound II, on the addition of fluoride compound I does not then appear in the system, and that a step in the overall reaction is isolated in which a decomposition of compound II occurs, accelerated in the presence of fluoride, giving the CcP-fluoride complex as the end product. Equally important is the conclusion that the system is not an equilibrium one, since the formation of compound II followed by its decomposition without any

further additions being made to the system (Fig. 6) means that at least one component is being used up. This cannot be the peroxidase because the spectrum of the fluoride complex appropriate to the concentrations used appears at the end of the experiment. Hence peroxide must be destroyed via the decomposition of compound II.

Fig. 6. Optical density changes at $420\text{ m}\mu$, the isobestic point for CcP and its fluoride complex, when H_2O_2 is added to give 1.2, 0.8 and $1.2\text{ }\mu\text{M}$ in a solution containing $0.76\text{ }\mu\text{M}$ -CcP and $53\text{ }\mu\text{M}$ -NaF, the addition being made at times 0, 1200 and 2000 sec., respectively.

The kinetics of this reaction were investigated by measuring optical density changes at $420\text{ m}\mu$. The reaction was found to be first order, as shown in Fig. 7, like the uncatalysed decomposition. At the highest fluoride concentration used, 7.5 mM , the velocity constant was 8.6 times greater than with no fluoride present, that is the half-reaction time was 50 sec. compared with about 7 min. The first-order constants were themselves a linear function of the fluoride concentration (Fig. 7, inset). This suggested that there might be a correlation between the velocity constant k_f for the reduction:

compound II + fluoride (+ reducing agent)

and the equilibrium constant for the formation of the fluoride complex

This was checked by comparing velocity constants and equilibrium constants at pH 5.3 and 7.0. The equilibrium constant is dependent on pH, varying approximately inversely with the hydrogen-ion

Fig. 7. The first-order decomposition of compound II at pH 7.0 in the presence of (a) 2.10 mM - and (b) 4.6 mM -NaF and the variation of k_{obs} , the first-order constant so obtained, with the concentration of NaF used.

concentration, and Table 4 shows the velocity constant to have the same type of variation, the ratio K_f/k_f being approximately constant. There thus appears to be a correlation between the affinity of the fluoride for the ferric haemoprotein and the velocity constant in the catalysed reduction of the higher oxidation state to the ferric state. In a similar way cyanide, which also has a high affinity for peroxidase, should also increase the rate of reduction of compound II. This was found to be so, although the experimental error in the determination of the velocity constants was greater because of the smaller optical density increment on going from compound II to the cyanide complex (George, 1953). The equilibrium constant for the formation of the cyanide complex was found to be relatively pH independent and k_{cyn} showed similar behaviour as shown in Table 4.

A further experiment was made using azide as the complexing agent which was found to have, at 30° , reasonably high equilibrium constants of

Table 4. Correlation between the velocity constants k_f and k_{cyn} for the catalysed decomposition of CcP compound II in the presence of fluoride or cyanide, and the equilibrium constants K_f and K_{cyn} for the fluoride and cyanide complex formation with the enzyme itself under the same experimental conditions, at 30°

pH	K_f (mole ⁻¹)	k_f (mole ⁻¹ sec. ⁻¹)	K_f/k_f (sec.)	K_{cyn} (mole ⁻¹)	k_{cyn} (mole ⁻¹ sec. ⁻¹)	$K_{\text{cyn}}/k_{\text{cyn}}$ (sec.)
5.3	1.3×10^5	32	4.1×10^3	2.45×10^5	16	1.5×10^4
7.0	4.2×10^3	2.3	1.8×10^3	4.4×10^5	10.2	4.3×10^4

$1.2 \times 10^4 \text{ M}^{-1}$ at pH 5.3 and $1.2 \times 10^3 \text{ M}^{-1}$ at pH 7.0, but no satisfactory indication of any increased rate of reduction could be obtained.

DISCUSSION

The oxidation-reduction properties of compound II established in the previous paper confirm the suggestion made recently (George, 1952*a, b*) that the transition from compound I to compound II is a reduction process, e.g.

where AH_2 represents either reducing groups present in the enzyme preparation or the deliberately added reducing agent. This mechanism explains why the transition is faster with some enzyme preparations than others for the amount and kind of trace reducing matter could vary, and it explains too why the transition is speeded up by all reducing agents that react with compound II (Chance, 1949*b*).

Although an equilibrium mechanism of the type

for the overall formation of compound II can account for a titration curve deviating from the ideal linear form obtained when a very stable complex is formed, such a mechanism is ruled out because the position of the titration curves in Figs. 1 and 2 is independent of the absolute concentration of the reactants, quite apart from the fact that the mechanism is incompatible with the one equivalent reduction of compound II. The experiment in which a further amount of CcP was added to a mixture of CcP and hydrogen peroxide, such that just 100% of compound II had been formed, showed that no peroxide was left to form more compound II, and therefore a set of irreversible reactions competing for peroxide or other oxidizing intermediates must be responsible for the titration behaviour observed. This conclusion in no way depends on the other feature of the titration curves, namely the apparent production of more than 1 mole of compound II from 1 mole of peroxide, which is also inexplicable in terms of the above equilibrium reaction. This behaviour was found with all the CcP samples and three out of four HRP samples, although the result in the case of HRP depends on the validity of the extrapolation used to determine the initial concentration of compound II. The fact that one HRP sample did not show this behaviour and that it was not previously observed with CcP may be due to different amounts of reducing matter being present in the preparations or reducing matter with a different activity. The varied properties this reducing matter may possess is shown up by the changing stability of compound II in the HRP systems as the preparations are kept,

and by the full formation of compound II with CcP in contrast to the 50 to 80% formation observed with HRP.

The titration curves suggest that in the limit 2 moles of compound II can be formed from 1 mole of peroxide, which would be possible in terms of oxidizing equivalents since peroxide possesses two oxidizing equivalents and compound II is one equivalent above the ferric state of the enzyme. For this to occur the net reaction

must also be thermodynamically possible, and the tentative values of the potentials of the two couples compound I/compound II and compound II/ferriperoxidase, which were given in Part I, are in agreement with this net reaction proceeding with a decrease in free energy corresponding to 0.3–0.6 v. However, it is clear that this net reaction is slow, or can only proceed via the reducing matter present, e.g.

otherwise 2 moles of compound II would invariably be formed for each mole of peroxide reacting. The observation that, with HRP, compound II is never formed fully except when reducing agents are deliberately added shows they play an essential role. A mechanism involving (b) and (d), together with other reactions competing for the AH radical, could account for the type of titration curve obtained. These considerations have an important bearing on the kinetics of peroxidase action. Since the production of compound II is not simply a function of the enzyme and peroxide concentrations but also involves a reducing agent there is no necessary correlation, like that which follows from the Michaelis-Menten enzyme-substrate complex mechanism, between the titration curve obtained in the absence of an added reducing agent and the kinetics of the system when one is present, since the added reducing agent may, in the latter case, be in part or entirely responsible for the reduction of compound I to compound II.

The decomposition of compound II

Since compound II is one oxidizing equivalent above ferriperoxidase there is no possibility of a dissociation like (c) above liberating ferriperoxidase, for no stable entity comprising half an oxygen atom or a hydrogen atom exists. This is a further distinction between compound II and the Michaelis-Menten type of enzyme-substrate complex. Compound II can only return to ferriperoxidase by reduction.

The spontaneous decomposition of compound II, although following first-order kinetics as shown in

Fig. 5 above, is presumably pseudo first order, for the magnitude of the velocity constant is dependent on the absolute concentration of compound II, that is compound II is more stable the lower its concentration. This would follow if the reducing matter bringing about its reduction were associated with the enzyme preparation either as trace impurities or as reducing groups on the enzyme protein itself such that the reducing group on one enzyme molecule reacts with the compound II group on another. Compound II formed from the CcP sample did not become more stable after the addition of a few equivalents of peroxide, unlike HRP (Chance, 1949*b*). A possible reason for this is the greater concentration of diluent protein, about 80%, providing a reservoir of reducing groups.

The experiments illustrated in Figs. 6 and 7 and Table 4 show that sodium fluoride brings about an increase in the rate of reduction of compound II giving as the reaction product the CcP-fluoride complex, and that potassium cyanide shows corresponding behaviour. Had cyanide alone shown this effect a likely explanation would have been the oxidation of cyanide ion to cyanogen for the oxidation-reduction potentials are favourable. But the observed hydrogen ion independence of the reaction is against this mechanism for in the pH range 5.3–7.0 an inverse relationship between the rate and the hydrogen-ion concentration should follow, arising from the increase in the cyanide-ion concentration. Oxidation of the undissociated hydrogen cyanide molecule would fit the results, but the similar behaviour found with fluoride calls for a more general mechanism since on energetic grounds the oxidation of either hydrogen fluoride or fluoride ions is ruled out. The correlation shown in Table 4 between the velocity constants k_f and k_{cym} and the equilibrium constants K_f and K_{cym} suggests that the velocity constant is larger when the overall free-energy change in the reduction process is larger. For example, the free-energy change in the reaction

compound II + e^- + fluoride $\rightarrow$ CcP-fluoride complex

is 7.1 kcal. more negative than the straight reduction in (a) at pH 5.3 and 30°. A possible mechanism for such an effect is a partial binding of fluoride in the activated complex lowering the free energy of activation. The pH dependencies of the equilibrium constants for the formation of the CcP fluoride and cyanide complexes are similar to those for HRP (Chance, 1952*a*) and favour a combination between CcP and the undissociated acid. The mechanism of this is not known with certainty. It is different from ferricytochrome *c* where the cyanide ion is bound (George & Tsou, 1952) and may be different from methaemoglobin, although there is some divergence of opinion as may be seen in

Lemberg & Legge's (1949) review of the earlier work and more recent data where the pH dependence of the dissociation of methaemoglobin-cyanide does not conform to that expected if the cyanide ion were bound to the iron (Chance, 1952*b*). Clearly a detailed mechanism for the decomposition of compound II with fluoride or cyanide present must await the solution of this structural problem.

In a recent paper Chance (1952*a*) reports qualitative observations on the effect of hydrochloric acid decreasing the stability of compound II in solutions of pH 2.0 which may involve a similar mechanism, since hydrochloric acid combines with HRP under these conditions.

A comparison of the peroxide reactions of peroxidase, catalase, and metmyoglobin

The knowledge of the oxidation-reduction properties of the intermediate compounds and the way they are formed from hydrogen peroxide affords an interesting comparison between these three haemoproteins. Keilin & Hartree (1945) showed the catalase-hydrogen peroxide system to be capable of oxidizing simple primary aliphatic alcohols to aldehydes with high catalytic efficiency. Peroxidase is unable to do this and likewise catalase does not bring about the rapid oxidation of phenols, aromatic amines, etc., which the peroxidase system oxidizes efficiently. Keilin & Mann (1937) found that peroxidase compound II reacted rapidly with these reducing agents, and in extensive kinetic studies Chance (1949*d*) showed that compound II is the rate-limiting intermediate in peroxidase reactions whereas only compound I is apparently involved in the rapid oxidation of alcohols by the catalase system. But a little compound II is formed in this reaction and Chance (1950) has also shown that compound II is produced in the catalase-ascorbic acid system. He suggested that it was an unreactive enzyme-substrate complex, which played no essential role in the overall reaction, the production of which merely resulted in less enzyme being available to catalyse the oxidation via the formation and reaction of compound I. More recently, however, George (1952*c*) has shown that catalase compound I can be converted to compound II by a number of reducing agents including phenols like *p*-cresol. Taken in all, these observations suggest an alternative explanation for the specificity of the two enzymes based on a difference of degree rather than kind, namely that in both systems compound II follows compound I but in the case of catalase, compound II preferentially reacts again with the partially oxidized reducing agent before the entities separate, thus simulating an over-all two-equivalent process, e.g.

compound I + $AH_2 \rightarrow$ [compound II.AH]

$\rightarrow$ catalase + A.

The small amount of compound II accumulating in the catalase-alcohol system could then be explained if, by diffusion, some did not undergo the further reaction with AH *in situ*.

Although different in spectroscopic properties from compound II of peroxidase, the metmyoglobin compound shows the same one-equivalent reduction to the ferrihaemoprotein and the same type of pH dependence in its reaction with ferrocyanide (George & Irvine, 1951, 1952). These chemical similarities and the fact that both the metmyoglobin compound and peroxidase compound II form when an equal concentration of peroxide is added to the haemoprotein suggest that these are the analogous compounds of the two haemoproteins and that the correspondence is not between the metmyoglobin compound and peroxidase compound III (which requires a large excess of peroxide for its formation) as proposed by Keilin & Hartree (1951) on the basis of spectroscopic characteristics. With such similar chemical properties it is more likely that the differences in physical properties arise from the residual haemoprotein structure, for instance the iron-protein link may involve a different atom in the two cases.

However, there is a very marked difference in the formation of the metmyoglobin compound compared to compound II of peroxidase, for the analogue of compound I does not appear to exist in the case of metmyoglobin. This is probably the reason for the different behaviour observed when peroxide is added to a mixture of the haemoprotein and ferrocyanide. As shown in Table 2, ferrocyanide does not compete at all effectively in the sequence of reactions leading to compound II. In contrast, George & Irvine (1951, 1952) found that with metmyoglobin one equivalent of ferrocyanide present in the mixture initially allowed the formation of one equivalent of the compound to occur on addition of 1 mole of peroxide, although if the compound were formed first, and then treated with the same amount of ferrocyanide, the compound was completely reduced. They explained this result by the formation of a transient oxidizing entity X along with the metmyoglobin compound

metmyoglobin + H₂O₂

(2 oxid. equiv.)

→ compound + X
(1 oxid. equiv.) (1 oxid. equiv.)

which could react with the ferrocyanide if present initially but which disappears by reacting with trace-reducing matter if the formation is allowed to proceed independently. A tentative chemical mechanism was put forward in which X is the OH radical and the intermediate compound has a ferryl ion structure

where Fe_m³⁺ represents the ferriprotoporphyrin iron atom of metmyoglobin. The apparent inability of metmyoglobin to form a compound analogous to compound I is evidence against compound I having the structure of an enzyme-substrate complex for there is no obvious reason why structures of the type Fe³⁺(H₂O₂) or Fe³⁺·O₂H⁻ should not be common to both classes of haemoprotein, in view of their comparable reactions with true complexing reagents like fluoride and cyanide, etc., where the physical properties show great similarities and the only difference lies in the hydrogen-ion dependency of their formation. The possibility that the energetics can favour the stabilization of an effective +5 oxidation state of iron in the haemoprotein enzymes but not in metmyoglobin offers a more profound basis for the differentiation.

The new mechanisms discussed above have a considerable bearing on the role of haemoproteins in biochemical systems under the influence of radiation and in the photosynthetic process. In discussing a hypothetical scheme for the reduction of oxygen to water by the interaction of an iron-containing oxidase, Rabinowitch (1945) made use of a resonance-stabilized intermediate Fe³⁺+O or Fe³⁺+O⁻ which can be seen to have the redox characteristics now established for the metmyoglobin compound and peroxidase compound II.

SUMMARY

1. Spectrophotometric titrations of horseradish peroxidase and cytochrome c peroxidase with hydrogen peroxide suggest that more than 1 mole of compound II can be formed for 1 mole of hydrogen peroxide, the figure approaching two at low values of the hydrogen peroxide/peroxidase concentration ratio. Further experiments established that compound II is not formed in any equilibrium system but in a series of irreversible reactions competing for the oxidizing capacity of the peroxide. Reducing groups present in the enzyme system play an essential role in these reactions.

2. Compound II cannot dissociate into ferriperoxidase since its iron atom has an effective oxidation number of +4: only reduction can regenerate ferriperoxidase. This process, which occurs spontaneously with trace-reducing matter present in the enzyme sample, is more rapid if sodium fluoride or potassium cyanide are added and the fluoride or cyanide complex of ferriperoxidase is the end product. There appears to be some correlation between the velocity constant for this enhanced reduction and the equilibrium constant for the fluoride or cyanide complex formation.

3. If a slight excess of peroxide is added to the fluoride complex, compound II is formed and then disappears with reformation of the fluoride complex.

With a larger excess of peroxide some compound I is also formed. The way in which this reaction occurs excludes an explanation based on shifting the position of an equilibrium in the system; instead, peroxide must be used up in irreversible reactions.

4. The peroxide reactions of metmyoglobin, peroxidase and catalase are discussed in terms of the

redox properties of the intermediate compounds and the way they are formed.

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REFERENCES

- Altschul, A. M., Abrams, R. & Hogness, T. R. (1942). *J. biol. Chem.* **136**, 777.
 Baeyer, A. & Villiger, V. (1901). *Ber. dtsch. chem. Ges.* **34**, 738.
 Chance, B. (1943). *J. biol. Chem.* **101**, 553.
 Chance, B. (1949*a*). *Science*, **109**, 204.
 Chance, B. (1949*b*). *Archiv. Biochem.* **22**, 224.
 Chance, B. (1949*c*). *Archiv. Biochem.* **21**, 416.
 Chance, B. (1949*d*). *J. biol. Chem.* **179**, 1341.
 Chance, B. (1950). *J. biol. Chem.* **182**, 649.
 Chance, B. (1951*a*). *Advanc. Enzymol.* **12**, 153.
 Chance, B. (1951*b*). *The Enzymes*, ed. by J. B. Sumner and K. Myrbäck. New York: Academic Press Inc.
 Chance, B. (1952*a*). *J. biol. Chem.* **194**, 483.
 Chance, B. (1952*b*). Unpublished results.
 George, P. (1952*a*). *Nature, Lond.*, **169**, 612.
 George, P. (1952*b*). *Advanc. Catalys.* **4**, 367.
 George, P. (1952*c*). *Biochem. J.* **52**, xix.
 George, P. (1953). *Biochem. J.* **54**, 267.
 George, P. & Irvine, D. H. (1951). *Nature, Lond.*, **168**, 164.
 George, P. & Irvine, D. H. (1952). *Biochem. J.* **52**, 511.
 George, P. & Tsou, C. L. (1952). *Biochem. J.* **50**, 440.
 Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 293.
 Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
 Keilin, D. & Mann, T. (1937). *Proc. Roy. Soc. B*, **122**, 119.
 Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*. New York: Interscience.
 Rabinowitch, E. I. (1945). *Photosynthesis and Related Processes*, p. 294. New York: Interscience.

The Reaction between Metmyoglobin and Alkyl Hydroperoxides

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In a previous paper, George & Irvine (1952) showed that the reaction between metmyoglobin and hydrogen peroxide was inconsistent with an equilibrium of the enzyme-substrate type, i.e. $E + S \rightleftharpoons ES$. In this reaction the metmyoglobin (MetMb) is oxidized by the peroxide, and the resultant compound is one in which iron is in the effective oxidation state of +4.

Since it was known that alkyl hydroperoxides behaved similarly to hydrogen peroxide in forming a red intermediate compound with methaemoglobin (Keilin & Hartree, 1935), the interactions of these peroxides and MetMb were investigated to see whether the MetMb underwent oxidation of the same type.

In this paper it is shown that spectroscopically the intermediate compounds formed by two alkyl hydroperoxides are the same, and identical with that produced by hydrogen peroxide. The experimental evidence supports a reaction mechanism similar to that suggested for the hydrogen peroxide reaction.

Nomenclature

In the previous papers (George & Irvine, 1951, 1952) the intermediate compound was referred to as a 'complex' partly to preserve continuity with the widespread use of this term for the intermediate compounds formed by peroxides in peroxidase and catalase systems, and partly because such compounds, independent of their chemical structure, may be regarded as co-ordination complexes of iron in the sense that more groups are bound to the iron atom than can be accounted for by the primary valencies of 2 and 3 in its ferrous and ferric states.

In enzyme chemistry however the term complex is usually identified with the enzyme-substrate complex of the Michaelis and Menten theory of enzyme action,

or with the modification in which the final reaction is written

The formation and subsequent reactions of the intermediate compound formed from metmyoglobin and peroxides do not correspond to the scheme of chemical