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

1. TITRATION WITH REDUCING AGENTS

By P. GEORGE
Department of Colloid Science, University of Cambridge

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The haemoprotein enzyme peroxidase, containing iron as ferriprotoporphyrin, catalyses very effectively the oxidation of fairly complicated organic molecules like phenols and amines by hydrogen peroxide. This action is intimately connected with its ability to form intermediate compounds with hydrogen peroxide in the absence of the reducing agent, for such compounds react very rapidly with the reducing agent if it is then added. Three such compounds recognizable by their colour and absorption spectrum can be formed depending on the experimental conditions, and they are related as follows:

$$\underset{\substack{1\text{ mole}\\(\text{brown})}}{\text{Peroxidase}} + H_2O_2 \rightarrow \underset{\substack{1\text{ mole}\\(\text{green})}}{\text{complex I}} \xrightarrow[\text{transition}]{\text{spontaneous}} \underset{(\text{pale red})}{\text{complex II}}$$

$$\underset{(\text{pale red})}{\text{complex II}} \xrightarrow{\text{excess } H_2O_2} \underset{(\text{deep red})}{\text{complex III}}$$

Following the successful application of the Michaelis-Menten enzyme-substrate complex theory to other enzymic reactions, these compounds have been regarded as enzyme-substrate complexes, peroxidase-H_2O_2 or peroxidase-OOH^- and named accordingly complexes I, II and III. The second and third compounds were first described by Keilin & Mann (1937) who showed that both could be reduced back to the ferric form of the enzyme. Theorell (1941) showed that complex II was not formed immediately when hydrogen peroxide was added to peroxidase but that a transient green-coloured compound, complex I, was formed first which very rapidly changed into complex II. Chance (1949*a*, 1951*b*, *c*) has carried out an extensive kinetic investigation of peroxidase action and interpreted the results in terms of the following Michaelis-Menten mechanism:

$$\text{Per.OH} + \text{HOOR} \rightleftharpoons \text{Per.OOR (I)} + H_2O, \quad (1)$$

$$\text{Per.OOR (I)} \rightarrow \text{Per.OOR (II)}, \quad (2)$$

$$\text{Per.OOR (II)} \xrightarrow{\text{spontaneous decay}} \text{Per.OH}, \quad (3)$$

$$\text{Per.OOR (II)} + AH_2 \rightarrow \text{Per.OH} + \text{ROH} + A. \quad (4)$$

This has been widely accepted and accounts also for the comparable catalytic activities of complexes formed with hydrogen peroxide and alkyl hydroperoxides of the type ROOH. The symbols Per.OH represent the ferric form of the enzyme with an OH group bound to the iron atom, and Per.OOR (I) and Per.OOR (II) the two enzyme-substrate complexes. The mechanism is a modification of that originally suggested by Michaelis & Menten in that the products are formed by the complex reacting with another molecule, AH_2, and not undergoing a unimolecular decomposition.

Such a mechanism has the following disadvantages:

(i) It gives no clue to differences in structure to account for the widely diverse spectroscopic and magnetic properties of the complexes. (ii) It is difficult to reconcile the type of chemical reaction shown in step (4), where four covalent bonds are broken and reformed, with the high speed of the reaction. (iii) It is difficult to envisage reaction (4) proceeding with a single electron transfer reducing agent, like ferrocytochrome *c*, without involving a two-stage reaction and consequent elaboration of the mechanism (see Chance, 1951*a*). (iv) It is difficult to understand the transition from complex I to complex II, in reaction (2), particularly in view of Chance's finding that the transition is speeded up by all reducing agents that react with complex II (1949*b*).

A mechanism avoiding all these disadvantages was suggested by George (1952*a*) which is based on the discovery by George & Irvine (1951, 1952) that the compound formed from metmyoglobin and hydrogen peroxide does not contain H_2O_2 or O_2H^- as a component part of its structure because the reduction of this compound back to metmyoglobin involves only one equivalent. The compound thus contains iron with an effective oxidation number of +4. If complex II of peroxidase is also of this type the transition from complex I to complex II is easily explained as a single equivalent reduction process and the paradox vanishes in understanding how a strong oxidizing agent, Per.OOR (I), changes into another strong oxidizing agent, Per.OOR (II),

Table 1. *Calculation of the equilibrium constant for the formation of the CcP-cyanide complex on the assumption that the CcP sample was lower in concentration than the value of 30 μM determined from d_{410}.*

Cyanide present (μM)	Observed percentage complex formed	Equilibrium constant × 10^{-5}, [CcP] in μM				
		30	28·0	27·5	27·0	20·0
14·1	45·7	12·1	5·30	4·68	4·25	1·01
20·8	64·0	8·2	5·10	4·66	4·35	2·10
27·5	78·0	6·8	5·30	4·98	4·95	2·78

in the presence of a reducing agent without chemical reaction occurring.

As yet the chemical structures of complexes I–III are unknown, and for this reason they are referred to in this paper as compounds I–III because the term complex implies that the structure contains H_2O_2 or O_2H^-. In the biochemical literature the oxidizable substrates are often termed hydrogen donors or electron donors: since this also suggests a preferred reaction mechanism, which still remains to be established, the more general term reducing agent is adopted here.

This paper presents evidence that compound II of horseradish peroxidase and cytochrome *c* peroxidase contain the iron in the quadrivalent state or an equivalent structure. A preliminary account of the experiments has already appeared (George, 1952*a*, *b*).

EXPERIMENTAL

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

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

In some chemical mechanisms the symbol Fe_p^{3+} has been used to denote the ferriprotoporphyrin iron atom in the enzyme. Its net charge within the co-ordination unit is really +1 due to replacement of two pyrrolic H atoms, but the use of Fe_p^{3+} facilitates comparison with ionic iron reactions and makes valency changes simpler to follow.

Methods. The reactions were carried out at room temperature, 28–32°, in 1 cm. cuvettes of a Beckman Model DU spectrophotometer and optical densities measured in the usual way. A.R. grade reagents were employed. Phosphate buffers of pH 5·3–5·4 and pH 7·0 were made by mixing the appropriate solutions of 0·067 M-KH_2PO_4 and 0·067 M-$Na_2HPO_4.2H_2O$ and diluting to give a total phosphate concentration of 0·01 M. Merck's Perhydrol was diluted to give H_2O_2 stock solutions of about 50 μM; the concentration was determined spectrophotometrically at 230 mμ. where mε = 0·0724.

A several-times recrystallized sample of HRP was used which had been prepared by Theorell's (1942) method; concentrations were calculated on the basis mε at 402 mμ. = 81 appropriate to this type of preparation. The ratio E_{402}/E_{280} was 2·7 indicating about 90% purity (Theorell & Maehly, 1950). Cytochrome *c* had been prepared by Keilin & Hartree's (1945) method.

Four different samples of CcP were used which were prepared by the method of Abrams, Altschul & Hogness (1940) except that the treatment with alumina gel was omitted. From their data the ratio of E_{410}/E_{270} for a pure sample can be estimated as 1·47 (Altschul, Abrams & Hogness, 1942). On this basis the present enzyme samples, 1, 2, 3 and 4 were only 23, 23, 11 and 14·5% pure. The contribution of the protein diluent to the optical density in the Soret region was obtained using sample 2 in the following experiment. A concentrated cyanide solution was added to the enzyme solution in 0·01 M-phosphate buffer, pH 7·0, which was nominally 30 μM with respect to CcP, as determined from E_{408}, taking mε = 93, according to the earlier workers. The cyanide complex has a broad absorption band at 544 mμ. in the visible region and the percentage of complex formed was obtained by averaging optical density increments at 535, 540 and 545 mμ., which agreed to within 1%. In Table 1 equilibrium constants are calculated from these data on the assumption that the enzyme concentration had the values 30·0, 28·0, 27·5, 27·0 and 20·0 μM. It can be seen that a value 27·5–28·0 μM gives the most concordant figure for the equilibrium constant, which shows that the enzyme concentration based simply on E_{410} is about 8% too high. This factor has been used in calculating the true enzyme concentration for all four samples, since the variation in the diluent protein concentration would only change this at most by 1% which is within experimental error. Enzyme activity tests were carried out for all four samples by observing the catalysis of the oxidation of ferrocytochrome *c* with hydrogen peroxide: this gave values between 5 and 10 times greater than that obtained by the earlier workers: this can only be attributed to idiosyncrasies in the test itself.

The absorption spectra in the Soret region of CcP and a number of its complexes which have been used in the present investigation are given in Figs. 1 and 2. The spectra of CcP and compound II are from mean values obtained using all four enzyme samples. For the other compounds sample 1 was used. At all wavelengths a contribution to the optical density by the diluent protein amounting to 8% of E_{410} has been assumed and the resulting optical densities adjusted according to E_{408} for 1·0 mM-CcP = 93 cm. The character of these absorption spectra, particularly the symmetry of the band of the ferro-CcP-CO complex, is a good indication that no other haemoproteins were present.

Experimental conditions for the study of compound II of HRP and CcP

The spontaneous decomposition of compound II is faster the higher the absolute concentration and so, to make the necessary corrections for this as low as possible, enzyme concentrations of 1–2 μM were usually used. Such solutions have optical densities of the order 0·080–0·160 in the Soret region, with optical density increments accompanying a chemical reaction of the order 0·060, permitting measurements with an accuracy of about 2%. The spon-

taneous decomposition is presumably the reaction of compound II with reducing matter present in the enzyme preparation either as trace impurities or actual reducing

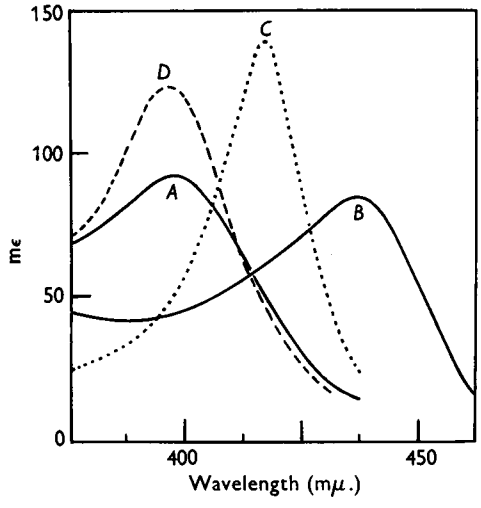

Fig. 1. Spectra of CcP and some of its derivatives. *A*, CcP; *B*, ferro-CcP; *C*, ferro-CcP-CO; *D*, CcP-fluoride.

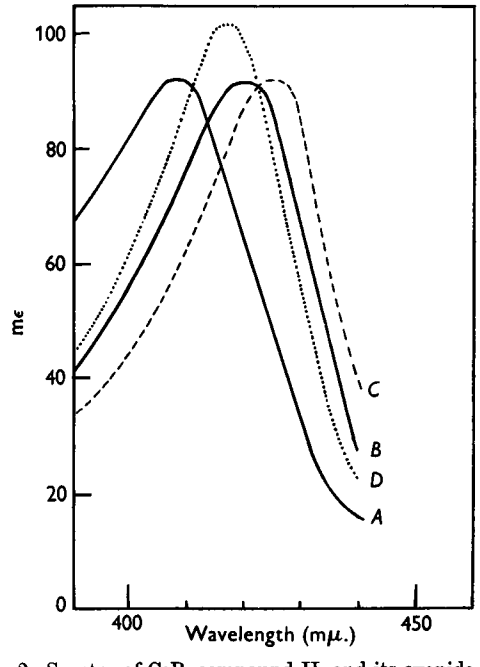

Fig. 2. Spectra of CcP, compound II, and its cyanide and azide complexes (*A*, *B*, *C* and *D* respectively).

groups on the enzyme molecule itself. There was a considerable difference between the samples of HRP and CcP in this respect. Compound II of CcP was found to be the more stable with a half-life of the order of 10–30 min. at 30° for a 1–3 μM solution. Compound II of HRP had a half-life of about 3–5 min. at the start of the investigation when the enzyme sample had been prepared for 3 months. Later, however, when the sample had been prepared for 6–9 months, the half-life had increased and the compound was as stable as that from CcP. Except with the first experiments with HRP, corrections for this spontaneous decomposition were negligible with both enzymes in the low concentration experiments.

Determination of the absorption spectrum of compound II

There is another stage in the peroxidase-H_2O_2 reaction where this differing reducing capacity has an effect and it again appeared that the HRP contained less reducing matter than the CcP. When H_2O_2 was added to CcP the Soret band, which has its maximum at 408 mμ. for the free enzyme, shifted toward the red with the maximum now at 420 mμ. shown in Fig. 2. Using 1 μM concentration this reaction was found to be complete by the time the first spectrophotometric measurement was made some 15 sec. after mixing. If about a fivefold molar excess of H_2O_2 was present and small amounts of a reducing agent like *p*-cresol added of the order of the enzyme concentration, no alteration was observed in the height or position of the Soret band of compound II. This was taken to mean that the transition from compound I to compound II was already complete since reducing agents speed the transition, and that the enzyme preparation itself contained enough reducing matter to effect the rapid transition. Further addition of *p*-cresol in excess of the H_2O_2 added reduced compound II back to the free enzyme, which was completely recovered within experimental error.

With the HRP under similar conditions the absorption band on adding peroxide had its maximum at about 420 mμ. but was less intense: the addition of small amounts of reducing agents caused the band to increase in intensity so that finally it had the same extinction coefficient as the free enzyme (Fig. 3). Evidently insufficient reducing matter was present in the enzyme sample to complete the transition from compound I to compound II. This change in spectrum is in accord with Chance's (1949*c*) data for the absorption spectrum of compound I, which has an absorption band of low intensity at 410 mμ., since a mixture of compounds I and II would give the type of spectrum shown in Fig. 3*A*. As in the case of CcP, reducing agents in excess were found to regenerate the enzyme quantitatively. The spectrum obtained by adding a small amount of *p*-cresol to HRP with a sixfold excess of H_2O_2 present has been taken to represent the full conversion of HRP into compound II, and concentrations of compound II have been obtained by optical density measurements at 420, 425, 430 and 435 mμ. taking as standard $\Delta\mu\epsilon_{425} = 0{\cdot}044$ for its reduction back to the free enzyme.

These results with CcP and HRP, showing that the band of compound II is of about the same intensity as that of the free enzyme, suggest that the spectrum reported by Altschul *et al.* (1942) is low by about 4% by reason of incomplete conversion from compound I to compound II which might well occur with a more pure preparation than the one used here. On this assumption $\Delta\mu\epsilon_{425}$ for full conversion is 0·0365. The values obtained with the present enzyme samples are somewhat higher: sample 1, 0·043; sample 2, 0·0415 and 0·048; sample 3, 0·0395 and sample 4, 0·0385.

A mean value of 0·0405 has been used in subsequent calculations. The reason for the discrepancy is unknown, but it is reasonable that $\Delta\epsilon_{425}$ for CcP should be less than that for HRP because the absorption bands of CcP and its compound II are closer together as a comparison of Figs. 2 and 3 shows.

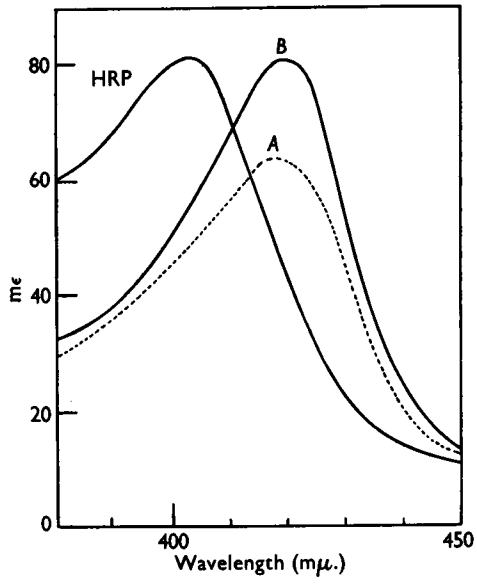

Fig. 3. Spectra of HRP and, *B*, compound II obtained by adding 1·9 μM-*p*-cresol to 1·6 μM-HRP with 6·0 μM-H_2O_2 present. Spectrum *A* is typical of HRP with a small excess of H_2O_2 but no reducing agent present—a mixture of compounds I and II (1·0 μM-HRP and 1–3 μM-H_2O_2).

Titration of compound II of CcP and HRP with potassium ferrocyanide

Preliminary experiments showed that the reaction of compound II with ferrocyanide ions resembles that of the metmyoglobin-H_2O_2 compound, for the reaction is faster in more acid solution (George & Irvine, 1952). At pH 7·0 the reaction had a half-time of about 15 min. with 1·4 μM-CcP compound II and 0·4 μM-$Fe(CN)_6^{4-}$, which is too slow to permit an accurate titration since the spontaneous decomposition of compound II had a half-time of about 30 min. under these conditions. At pH 5·4, however, the reaction was much faster (Fig. 4). The velocity constant for the reaction can be computed as about 10^6 M^{-1} sec.$^{-1}$

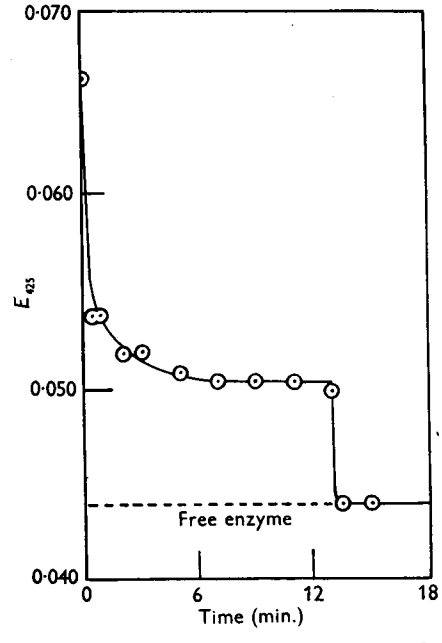

Fig. 4. Optical density changes at 425 mμ. when two portions of ferrocyanide giving 0·40 μM are added to a mixture of CcP and compound II at times 0 and 13 min. respectively, in a solution of pH 5·4.

and a rough comparison of the data shows the reaction rate to vary according to at least the first power of the hydrogen-ion concentration. The curve in Fig. 4 shows that an optical density change of 0·016 cm.$^{-1}$ at 425 mμ. accompanies the reaction of compound II with 0·04 μM-$Fe(CN)_6^{4-}$, i.e. 0·016/(0·40 × 0·0405) = 1 mole of compound II reacts with 1 mole $Fe(CN)_6^{4-}$. The results of similar experiments are given in Table 2 and all show that the reduction of compound II to the free enzyme involves only one oxidizing equivalent.

A satisfactory titration of compound II of HRP was more difficult, for, as shown above, complete

Table 2. *Titration of CcP compound II with ferrocyanide at* pH 5·4, *obtained by optical density measurements at* 425 mμ.

Ferrocyanide concentration (μM)	ΔE_{425}	Compound II reacting (μM)	Compound II / Ferrocyanide
0·15	0·0065	0·160	1·07
0·24	0·009	0·22	0·92
0·40	0·016	0·395	1·00
0·56	0·020	0·495	0·88
0·75	0·030	0·74	0·99
1·00	0·0365	0·90	0·90
		Mean	0·96 ± 0·60

Table 3. *Titration of HRP compound II with ferrocyanide at* pH 5·4, *obtained by optical density measurements at* 425 mμ.

Ferrocyanide concentration (μM)	ΔE_{425}	Compound II reacting (μM)	Compound II / Ferrocyanide
0·15	0·006	0·14	0·93
0·30	0·015	0·34	1·13
0·50	0·022	0·50	1·00
0·70	0·027	0·62	0·89
0·75	0·028	0·64	0·85
0·90	0·031	0·71	0·79
0·90	0·035	0·79	0·88
		Mean	0·92±0·08

conversion of the enzyme into compound II is only effected by adding small amounts of reducing agents with excess H_2O_2 present. These conditions are clearly incompatible with a titration. In the experiments below, the full amount of compound II was allowed to form, using a H_2O_2 concentration insufficient to give the maximum optical density change to ensure that no excess H_2O_2 was left; ferrocyanide was then added, and the titration result obtained interpreted on the basis that a little compound I was present.

Compound II of HRP was found to react more rapidly with ferrocyanide than CcP compound II, and the optical density of the solution after mixing was constant after about 40 sec. Table 3 sets out the results of several titrations calculated on the basis that only free HRP and compound II were present, showing that 0·92 mole of compound II reacts with 1 mole of ferrocyanide. An upper estimate of the amount of compound I actually present can be obtained from the observation that with 1 μM-HRP, $E_{425}=0{\cdot}029$ and the optical density after adding excess H_2O_2 to give the maximum change was 0·066. For 1 μM compound I and compound II, $E_{425}=0{\cdot}025$ and 0·073, hence about 14% of the HRP was present as compound I under these conditions. In the titration experiments where less H_2O_2 was used, a smaller fraction of compound I will have been present but it cannot be determined from these data; yet, whatever it might have been, it will raise the stoicheiometric factor of the reaction of compound II with ferrocyanide. Calculation shows that some 6% of compound I present could raise the stoicheiometric factor by about 0·09 and so it is justifiable to conclude that the factor is really unity.

It was observed that a freshly made solution of ferrocyanide did not react as rapidly as one that had stood for a few hours. The probable explanation of this is the formation of a small amount of $Fe(CN)_5H_2O^{3-}$ in the solution which reacts faster with compound II, permitting a catalysis of the ferrocyanide-compound II reaction through the subsequent reaction of $Fe(CN)_5H_2O^{3-}$ with $Fe(CN)_6^{4-}$. There was no evidence of any irreversible change taking place in such ferrocyanide solutions that might have altered the reducing capacity and so invalidate the titration results. This is borne out by similar results obtained using other reducing agents.

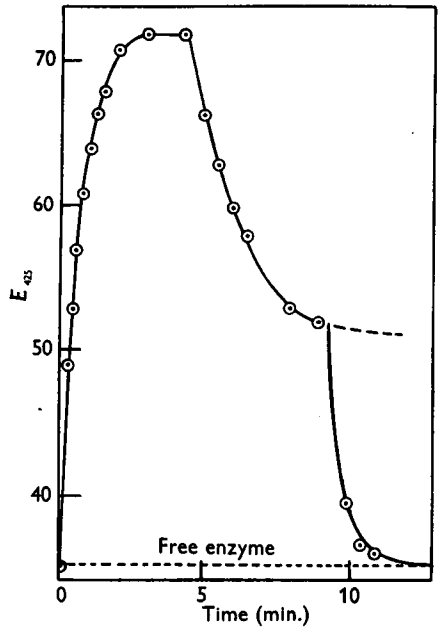

Fig. 5. Optical density changes at 425 mμ. when two portions of ferrous ion giving 0·5 and 1·0 μM are added to a mixture of HRP and compound II at times 4·3 and 9·3 min. respectively, in a solution of pH 5·4.

Titration of HRP compound II with ferrous ion

Spectrophotometric examination showed that a fivefold excess of Fe^{2+} reduced compound II quantitatively to the free enzyme at pH 5·4. The reaction is, however, slower than with ferrocyanide as shown in Fig. 5, from which the velocity constant can be estimated as about $10^4\ M^{-1}\,sec.^{-1}$. An interesting feature appearing in Fig. 5 is the relatively slow formation of compound II obtained after the enzyme sample was about 6 months old. The concomitant stability of the compound once

Table 4. *Titration of HRP compound II with ferrous ions at* pH 5·4, *obtained by optical density measurements at* 425 mμ.

Ferrous ion concentration (μM)	ΔE_{425}	Compound II reacting (μM)	Compound II / Ferrous ion
0·5	0·021	0·48	0·96
0·7	0·025	0·57	0·82
1·0	0·035	0·80	0·80
		Mean	0·86 ± 0·07

formed was shown by the optical density remaining constant for at least 2 min. This enabled titration with Fe^{2+} to be carried out, for the reduction took about 5 min. under these conditions. From Fig. 5 it appears that an optical density change of 0·021 at 425 mμ. occurs when compound II reacts with 0·05 μM-Fe^{2+}, i.e. 0·48/0·5 = 0·96 mole compound II reacts with 1 mole Fe^{2+}. The results of two other titrations are included in Table 4 giving a mean stoicheiometric factor of 0·86. Correction for the amount of compound I present would raise this figure and, since the slow formation of compound II indicates less active reducing matter in the system, it is likely that more compound I was present here than in the ferrocyanide ion titration. It is thus justifiable to regard the two titrations as being in substantial agreement with a stoicheiometric factor of unity.

Titration of CcP compound II with ferrocytochrome c

In the titrations with ferrocyanide and ferrous ion the reasonable assumption was made that compound II, present in excess, reacted with the full amount added to give ferricyanide and ferric ion. Ferrocytochrome *c* has the added advantage that its concentration changes can be measured spectrophotometrically, affording an additional check on the stoicheiometry.

In the following experiment H_2O_2 was added to a 1·50 μM solution of CcP giving 1·34 μM concentrations of compound II (see note later on concentrations), 0·05 ml. of a concentrated cytochrome *c* solution, in which 71 % of the pigment was in the reduced state, was then added so that the resulting solution was 1·58 μM in cytochrome *c*. Optical densities were measured at 413 and 433 mμ., the isosbestic points for CcP-compound II and ferri-ferrocytochrome *c* respectively, before and after the addition of cytochrome *c*. The reaction between compound II and ferrocytochrome *c* was observed to be very rapid, being complete by the time the first optical density was measured 15 sec. after the addition. The stoicheiometry of the reaction was calculated as follows.

Table 5 gives the optical density changes observed. At 433 mμ. the optical density of μM-cytochrome *c* was 0·0285 and the increment for conversion of CcP into compound II 0·0295: thus the contribution of cytochrome *c* to the optical density of the final solution 0·101 was 1·58 × 0·0285 = 0·045. So the CcP in this solution contributed (0·101–0·045) = 0·056. The optical density change accompanying the reaction of compound II with ferrocytochrome *c* was therefore (0·0875–0·056) = 0·0315, corresponding to 0·0315/0·0295 = 1·07 μM-compound II.

Table 5. *Data for the titration of CcP compound II with ferrocytochrome* c

Solution	Optical densities 410 mμ.	413 mμ.	433 mμ.
1·50 μM-CcP alone	0·140	0·131	0·048
With 2 μM-H_2O_2 present	—	0·131	0·0875
With 1·58 μM-cytochrome *c* then added (71 % reduced)	—	0·292	0·101
The same cytochrome *c* solution alone	—	0·190	0·045

At 413 mμ. the contribution of CcP and compound II to the optical density was 0·131 which agrees well with the estimated value of 0·132 obtained from the concentration 1·50 μM and a previously determined value of $\mu\varepsilon$ 0·088. The cytochrome *c* in the final solution thus had an optical density at 413 mμ. of 0·292–0·132 = 0·160. If no oxidation had occurred the optical density would have been 0·190, hence a change of 0·030 accompanied the oxidation by compound II.

The optical density increment for the oxidation of ferrocytochrome *c* to ferricytochrome *c* at 413 mμ. was found to be $\Delta\mu\varepsilon$ = 0·288, hence this change corresponds to the formation of 0·030/0·0280 = 1·05 μM ferricytochrome *c*. The original concentration of ferrocytochrome *c* in the sample was 0·71 × 1·58 μM = 1·12 μM and, since an excess of compound II was added, this full amount was available for oxidation. Thus these results show that

1·07 μM-compound II + 1·12 μM-ferrocytochrome *c* → 1·07 μM-CcP + 1·05 μM-ferricytochrome *c*.

It is clear that the stoicheiometric factor is unity and the deviation attributable to experimental error. In the calculation the observed optical densities at 410 and 413 mμ. have been assumed to be entirely due to CcP, neglecting the fact that about 8 % is due to the protein diluent because this does not affect the stages of the calculation when the total optical density is involved. The appropriate value of the optical density increment at 433 mμ. for the conversion of CcP to compound II has been obtained from the spectra on the basis that the value at 425 mμ. is 0·0405 as has been used in the previous titrations.

An attempt to bracket the oxidation-reduction potential for the reduction of compound II to the free enzyme

In a survey of the reaction of the secondary compounds with a variety of reducing agents it was found that both were reduced by chloroiridite ions with complete regeneration of the free enzyme as in ferrocyanide or ferrous ion reduction, although a larger amount was required in the case of CcP compound II. No reduction was observed with ferrous tris-2:2′-dipyridyl or tris-*o*-phenanthroline ions, but in the case of HRP where some compound I was present with compound II an increase in the intensity of the absorption band was observed like that shown in Fig. 3 which occurred when *p*-cresol was added, suggesting that these ferrous complexes can reduce compound I to compound II.

DISCUSSION

The nature of compound II

The titration of compound II with the various reducing agents all showed that it is reduced to ferriperoxidase Fe_p^{3+} in a one equivalent reduction:

$$\text{compound II} + e^+ \rightarrow Fe_p^{3+}. \quad \text{(reaction } a\text{)}$$

The compound thus has only one oxidizing equivalent in a reaction regenerating the free enzyme compared with the two oxidizing equivalents originally present in H_2O_2. This result requires that H_2O_2 or its anion O_2H^- cannot be a component part of the structure of compound II and so it should no longer be regarded as an enzyme-substrate complex (Chance, 1943, 1949*a–c*, 1951*b*, *c*). An iron compound whose oxidation-reduction reactions are expressed by reaction (*a*) formally contains iron with an oxidation number of +4. Several possibilities may be considered for the structure of such a compound: (i) simple quadrivalent iron Fe^{4+} bound in the porphyrin ring either covalently, or ionically as Fe^{2+}, and Fe^{3+} are bound in ferro- and ferri-haemoproteins; (ii) a derivative of quadrivalent iron with, for instance, O^- bound as in the structure FeO^{2+}, which in the case of ionic iron would be the ferryl ion and correspond to the vanadyl ion VO^{2+}; (iii) a biradical structure in which tervalent iron forms 'one end' of the radical, the other end being a normal radical grouping at a methine carbon atom, a pyrrolic carbon atom or some other atom within the conjugated network of porphyrin ring and haemoprotein linkage; (iv) a higher oxidation state of the haematin group in which the electron has been removed from a π-orbital common to the ring as a whole. The existence of this type of oxidation state has recently been demonstrated by Cahill & Taube (1951) for the structurally similar metal phthalocyanines containing copper, iron, cobalt, zinc or aluminium in tetrasulphonated derivatives. Structures (i) and (iv) arise by simple electron transfer and only differ as to which is the lower lying energy level, whereas structures (ii) and (iii) require bond-breaking reactions both in the formation and subsequent reaction of the compound. The magnetic susceptibility of compound II formed from H_2O_2 has not been measured, but that from CH_3OOH, which has an identical spectrum and similar titration curve (George, 1953) and may therefore be regarded as the same compound, has $\chi_m = 3500 \times 10^{-6}$ c.g.s. units (Theorell, Ehrenburg & Chance, 1953). A quadrivalent iron compound in which the bonds are essentially covalent requires two unpaired electrons per iron atom and this value for χ_m is a little greater than the theoretical value of 3390×10^{-6} c.g.s. units calculated for two unpaired electrons on a 'spin only' basis. The susceptibility value is therefore in accord with the oxidation-reduction behaviour of the compound as represented in reaction *a*, although Theorell, Ehrenburg & Chance choose to regard it as a ferric complex analogous to ferriperoxidase cyanide which has a susceptibility $\chi_m = 2970 \times 10^{-6}$ c.g.s. units.* The susceptibility value does not help to decide between the four structures for compound II given above except to rule out a purely ionic type of binding. The following chemical evidence does, however, suggest that bond-breaking reactions, as contrasted to simple electron transfer, are involved in the reactions of compound II.

First the reduction by ferrocyanide ion, or $Fe(CN)_5H_2O^{3-}$, is faster the higher the hydrogen-ion concentration, as shown above. Secondly, the reaction of compound II with ionizable reducing agents like quinol, guaiacol and pyrogallol is independent of pH in the range 3·6–6·7 (Chance, 1949*a*) suggesting that the conjugate acid of these reducing agents is the reacting species. Thirdly, the autoxidation of these same reducing agents is faster in more alkaline solution which is usually attributed to a preferred electron transfer mechanism involving an anionic species. The contrast between these two systems and the hydrogen-ion dependency of the ferrocyanide reaction both favour the inclusion of hydrogen in the reduction of compound II. This can most simply be illustrated with a 'ferryl ion' structure:

$$Fe_pO^{2+} + H^+ + e^- \rightarrow Fe_p^{3+} + OH^-,$$
$$Fe_pO^{2+} + AH_2 \rightarrow Fe_p^{3+} + OH^- + AH,$$

but similar reactions can be written for the 'biradical' structure.

* Theorell, Ehrenburg & Chance (1952) more recently report a revised value of $\chi_m = 4850 \times 10^{-6}$ c.g.s. units for compound II formed from CH_3OOH. They no longer propose that it is a simple ferric complex like the cyanide complex, but discuss its susceptibility in terms of the ferryl ion structure suggested by George & Irvine (1951).

A tentative value of about $-1{\cdot}0$ V. (U.S.A. convention) or the compound II/ferriperoxidase couple is afforded by the observation that compound II is reduced by chloroiridite ions but not by ferrous tris-2:2′-dipyridyl or tris-*o*-phenanthroline ions which have oxidation-reduction potentials of $-1{\cdot}02$, $-1{\cdot}06$ and $-1{\cdot}14$ V. respectively (Hume & Kolthoff, 1943; Walden, Hammett & Chapman, 1933; Woo, 1931). The uncertainty arises because little is known of the chloroiridate-chloroiridate system in other than acid solution and because a difference in charge type in the case of the ferrous complexes might result in a decrease in reaction rate obscuring the fact that a reaction can occur. This latter factor is probably absent, however, since there is evidence that compound I does react with the ferrous complexes.

The formation of compound II

The suggestion was made recently that the transition from compound I to compound II is a reduction process (George, 1952*a*, *b*)

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + AH. \qquad \text{(reaction } b\text{)}$$

Such a mechanism resolves the difficulty encountered with the enzyme-substrate complex theory in understanding how the transition is affected by trace-reducing matter in the enzyme samples and is speeded up by all reducing agents that react with compound II (Chance, 1949*b*).

According to this new mechanism compound II should be one oxidizing equivalent above the ferriperoxidase and this has now been established by the titrations with reducing agents. In addition, compound I should be two oxidizing equivalents above ferriperoxidase and so contain the original oxidizing capacity of the peroxide, as in fact has always been assumed in enzyme-substrate complex mechanisms. It is difficult to see how the two stages of the overall reaction could otherwise be accounted for in view of the oxidation-reduction properties of compound II; however, several of the experiments described above furnish evidence in support of this.

The spectra in Figs. 2 and 3 show that far less compound I is left in the final solution when peroxide is added to CcP as compared to HRP. The compound II titration data in Tables 2-5 show the stoicheiometric factor for the reduction of the CcP compound II to be nearly unity, whilst that for the HRP compound II is significantly lower. Reducing agents are known to speed the transition from compound I to compound II and so the only consistent explanation on the basis that both CcP and HRP secondary compounds have the same oxidation-reduction properties is that some reducing agent is used in the HRP experiments to convert compound I into compound II. This requires compound I to have a greater oxidizing capacity than compound II.

The experiments described in the next paper of this series (George, 1953) in which peroxide was added to a mixture of CcP and ferrocyanide show clearly that ferrocyanide is involved in oxidation-reduction competition reactions leading to the formation of compound II. More of the oxidizing capacity of the peroxide in the system can be accounted for under these conditions and the amount of ferrocyanide used in these preliminary reactions is approximately proportional to its initial concentration. This result would be expected according to reaction *b*. Following on these experiments Chance (1952) has shown that compound I can be reduced by ferrocyanide to compound II in a 1:1 reaction, by using a more acid solution where compound I is more stable, and employing a rapid-recording spectrophotometer.

The observation that the ferrous tris-2:2′-dipyridyl and tris-*o*-phenanthroline complexes give more compound II when added to a mixture of compound I and compound II without reducing compound II is further indirect evidence in favour. On the basis of reaction *b*, compound I but not compound II could have a high enough oxidation-reduction potential to oxidize the ferrous complexes.

A provisional value for the oxidation-reduction potential of the compound I–compound II couple may be obtained from the following reactions:

$$\text{peroxidase} + H_2O_2 \rightarrow \text{compound I},$$
$$\text{compound I} + H^+ + e^- \rightarrow \text{compound II} + H_2O,$$
$$\text{compound II} + H^+ + e^- \rightarrow \text{peroxidase} + H_2O,$$
$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O,$$

where H^+ is included arbitrarily to balance the overall reaction. This has an oxidation-reduction potential $E^\circ = -1{\cdot}77$ V. which becomes $-1{\cdot}44$ V. at pH 5·3 which was used in the experiments. The equilibrium constant and hence the free-energy change accompanying the formation of compound I from H_2O_2 is not known with certainty (Chance, 1949*b*), but for methyl hydroperoxide an equilibrium constant of $3{\cdot}1 \times 10^5\ \text{M}^{-1}$ is given. This is a reasonable lower limit for the constant using hydrogen peroxide and calculation from the above equations on this assumption gives $-1{\cdot}6$ V. for the oxidation-reduction potential of the compound I/compound II couple. No upper estimate of the equilibrium constant can be made, but even if it were as high as $10^{10}\ \text{M}^{-1}$ the potential would only fall to $-1{\cdot}3$ V. These rough calculations based on the new mechanism thus indicate that compound I could be a more powerful oxidizing agent than compound II by about 0·3–0·6 V. in accord with the observed reduction of compound I but not compound II by the ferrous complexes.

The mechanism of peroxidase action when compound II is involved

Chance's assumption that compound II is an enzyme-substrate complex capable of reacting thus:

$$\text{Per.OOH} - \text{II} + AH_2 \rightarrow \text{Per.OH} + H_2O + A$$

was based on measurements with excess reducing agent present showing that the decomposition of compound II was first order and the observed velocity constant directly proportional to the concentration of reducing agent (Chance, 1949*a*, *d*). With conditions giving a first-order reaction of this kind the stoicheiometry is obscured and his data fit equally well the reactions

$$\text{compound II} + AH_2 \xrightarrow[\text{determining}]{\text{rate}} \text{peroxidase} + AH$$

followed by $2AH \rightarrow A + AH_2$,

or $\text{compound II} + AH \rightarrow \text{peroxidase} + A$,

resulting in an over-all reaction of the type

$$2\ \text{compound II} + AH_2 \rightarrow 2\ \text{peroxidase} + A$$

in conformity with the titration results reported in this paper. This new mechanism is of particular importance in the oxidation of ferrocytochrome *c* by the peroxidase system for it resolves the difficulty in formulating a reaction with compound II on a two equivalent basis (Chance, 1951*a*).

In view of the wide acceptance of compound II as an enzyme-substrate complex it is desirable to summarize its properties revealed by the above experiments which serve to distinguish it from a true complex of this kind. (1) The substrate, peroxide, is not a component of its structure. (2) It cannot dissociate to give the free enzyme and a valency-saturated product: it can only be reduced to the free enzyme.

Chance has shown that compound II can behave kinetically as a rate-limiting intermediate in peroxidase reactions, but it does not necessarily follow that a Michaelis-Menten mechanism suitably modified to allow for a bimolecular reaction between Per.OOH-II and AH_2 is operative. Rate-limiting intermediates are always found in catalysed oxidation-reduction reactions, although the initial state of the catalyst usually predominates in the stationary state.

In formulating a chemical mechanism for peroxidase action (as distinct from a complete kinetic mechanism) it is now essential to consider possible structures for compound I in relation to compound II, which is an intermediate that can now be recognized as belonging to the same class as a free radical or a semi-quinone, for it permits a stepwise mechanism with the transfer of 1 oxidizing equivalent at each step. In general, compound I may either still contain H_2O_2 or its anion O_2H^- in an ion-pair complex and fulfil the structural requirements of an enzyme-substrate complex, or it may possess the same oxidizing capacity as H_2O_2 in the form of a higher oxidation state of iron, in this case +5, or in some equivalent structure like Fe_pO^{3+}. The formation of compound II can be accounted for equally well in both mechanisms, as may be seen in the following reactions in which compound II is assumed to have the ferryl ion structure, Fe_pO^{2+}:

(i) compound I as an ion-pair complex:

$$Fe_p^{3+}O_2H^- + AH_2 \rightarrow Fe_pO^{2+} + AH + H_2O;$$

(ii) compound I as a higher valency state:

$$Fe_p^{3+} + H_2O_2 \rightarrow Fe_pO^{3+} + H_2O,$$

$$\underset{\text{(I)}}{Fe_pO^{3+}} + AH_2 \rightarrow \underset{\text{(II)}}{Fe_pO^{2+}} + AH + H^+,$$

where Fe_p^{3+} represents the peroxidase iron atom.

It is not yet possible to decide to which class compound I belongs. There is no direct evidence that it is an enzyme-substrate complex capable of dissociating into free peroxidase and peroxide. On the contrary, George's (1952*b*) observations that peroxidases react with other oxidizing agents like HOCl, HOBr, BrO_3 periodate, ClO_2, ClO_2, ozone and persulphate in the presence of silver, but not cupric ions, favour the higher oxidation state theory. These experiments showed clearly that compound II was formed and the type of spectrum resembled that of a mixture with compound I as in Fig. 3*A*.

The formulation of a kinetic mechanism with reducing agents of the type AH_2 involves assumptions about the fate of the AH radicals. There is in addition the possibility that in the reactions for which data are available molecular oxygen was also a reactant. Even with ferrocytochrome *c* as the reducing agent unexpected side reactions occur. Chance (1950) found HRP and CcP were readily inactivated by the turnover of several separate additions of ferrocytochrome *c* and that with HRP the activity could be restored by the addition of a little *p*-phenylenediamine. These observations suggest that the reducing groups associated with the enzyme preparation play a dominant kinetic role, and until more is known of this behaviour no satisfactory kinetic mechanism can be advanced.

SUMMARY

1. Spectrophotometric titrations of compound II formed by interaction of peroxidase and hydrogen peroxide with reducing agents such as ferrocyanide, ferrocytochrome *c* and ferrous ions, show it to undergo a 1 equivalent reduction to ferriperoxidase, the initial oxidation state of the enzyme. Hydrogen peroxide or the anion O_2H^- cannot therefore be a component part of its structure and it should no longer be regarded as an enzyme-substrate complex,

Per.OOH, but as a compound in which the iron has an effective oxidation number of +4.

2. These results strongly support the following new mechanism for the interrelationship of compound I and compound II which necessitates a complete revision of the accepted mechanism for peroxidase action.

$$\text{Peroxidase} + H_2O_2 \rightarrow \underset{\text{(2 oxid. equiv.)}}{\text{compound I}} \xrightarrow[\text{reduction}]{\text{1 equiv.}} \underset{\text{(1 oxid. equiv.)}}{\text{compound II}}$$

(2 oxid. equiv.)

$$\underset{\text{(1 oxid. equiv.)}}{\text{compound II}} \xrightarrow[\text{reduction}]{\text{1 equiv.}} \text{peroxidase}$$

3. Preliminary values of 1·3–1·6 and approx. 1·0 V. have been estimated for the oxidation-reduction potentials of the compound I/compound II and compound II/peroxidase couples respectively at pH 5·3 (European convention).

4. Four types of possible chemical structure for compound II containing iron with an effective oxidation number of +4 are discussed, together with two general structures available for compound I, either as a ferric complex or a derivative containing iron with an effective oxidation number of +5.

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Comparative Detoxication

1. THE METABOLISM OF SULPHADIMIDINE IN THE LOCUST

By CELIA M. MYERS and J. N. SMITH
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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Knowledge of the 'detoxication mechanisms' of higher animals is finding useful application in the design of more effective drugs (cf. Brodie, 1952; Williams, 1951), and it is possible that similar studies in insects may lead to more effective insecticides (cf. Brown, 1951). Very little is known, however, of the metabolic changes undergone by foreign organic compounds in the insect. Most of the information available deals with the dehydrohalogenation of halogenated compounds such as DDT (1:1:1-trichloro-2:2-di(*p*-chlorophenyl)ethane) (Perry & Hoskins, 1950; Ferguson & Kearns, 1949; Winteringham, Loveday & Harrison, 1951), but apart from this there are only a few isolated reports of metabolic changes in insects, for example, oxidation of salicin to salicylaldehyde and salicylic acid in